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

FEDERAL SERVICE FOR VETERINARY AND PHYTOSANITARY SURVEILLANCE (ROSSELKHOZNADZOR)

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Congratulations on Russia Day

Dear readers! Please accept my sincere congratulations on the main state holiday – Russia Day!

This holiday symbolizes national unity and our common responsibility for the present and future of our country. June 12 is a significant day for every true patriot proud of the centuries-old history of their country, spiritual and cultural heritage, who works tirelessly for its prosperity.

Russia Day is a holiday of love and respect for our Motherland, the symbol of national unity. For every person, the Motherland begins where they were born, where they live, study and work. The present and the future of our country depends on each of us, on our common efforts and actions, intellectual and creative achievements. Only through joint efforts will we be able to overcome any challenges and achieve even better results.

On this solemn day, we all feel special pride for our great power and our compatriots.

On this festive day, let me wish you health and success in achieving your goals. May you be surrounded by comfort and wealth, happiness and mutual understanding, accompanied by peace, harmony and confidence in the future, and may the generosity of our land bring you prosperity and well-being.

I wish successful development in every sphere of activity both for Russia itself and for all its citizens. Let every citizen have a place in their soul for love for their homeland, and let the strength of the spirit of our ancestors bring development and give strength for great achievements as well as faith in a wonderful future.

Director of the FGBI "ARRIAH" *R. N. Rybin*

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In the course of my work, I unexpectedly discovered some areas that had not been seriously discussed yet...
Frank Burnet

African swine fever: one hundred years later

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SUMMARY
This brief report pays tribute to Robert Eustace Montgomery (1880–1932) whose name is associated with the discovery of African swine fever (ASF). A hundred years ago, he published a major report on study of new highly dangerous disease carried out in 1900–1917 in the East Africa. The infection fundamentals have been established and described – the etiological, immunological and nosological uniqueness of the infection, fatal susceptibility of domestic pigs, clinical sings and pathomorphology, viral etiology, natural reservoir and source of the infection, survival of the virus outside the body, many specific epizootological aspects. Taking into account current high publication activity it has been concluded that there is a large body of multi-faceted research focused on African swine fever. Gap analysis carried out by the large team of the European researchers and experts revealed the most challenging aspects – wild boars, ASF survival and transmission, biosecurity and surveillance. In addition to the gaps mentioned in these conclusions and recommendations there are serious gaps in African swine fever immunology, namely in protective immunity mechanisms, virus-macrophage interaction, *in vitro* virus phenotypic signs correlating with its virulence, etc. Evidently, it is hardly possible to expect development of anti-ASF vaccines and particularly the vaccines capable of preventing and effectively protecting against ASF epizooty according to the general understanding without addressing these issues.

Keywords: review, R. Eu. Montgomery, African swine fever, current panzooty, Gap analysis

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Африканская чума свиней через сто лет

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РЕЗЮМЕ
В кратком сообщении воздается должное Роберту Юстасу Монтгомери (1880–1932), с именем которого связано открытие африканской чумы свиней. Сто лет назад им опубликован масштабный отчет об исследованиях этого нового особо опасного заболевания, проведенных в 1900–1917 гг. в Восточной Африке. Установлены и описаны основополагающие положения – этиологическая, иммунологическая и нозологическая самостоятельность данной инфекции, фатальная восприимчивость домашних свиней, клиника и патоморфология, вирусная этиология, природный резервуар и источник заражения, устойчивость вируса вне организма, многие частные элементы эпизоотологии. Применительно к текущей ситуации на основании высокой публикационной активности сделано заключение о чрезвычайном массиве разносторонних исследований по африканской чуме свиней. GAP-анализ (анализ пробелов, Gap analysis), выполненный большим коллективом европейских ученых и специалистов, позволил определить наиболее проблемные вопросы: дикие кабаны, выживание после африканской чумы свиней и трансмиссия, биобезопасность и надзор. Помимо отмеченных в этих выводах и рекомендациях, серьезные пробелы существуют в иммунологии африканской чумы свиней, в частности, относительно механизмов протективного иммунитета, взаимодействия вирус – макрофаг, фенотипических признаков вируса *in vitro*, коррелирующих с вирулентностью, и другие. Очевидно, что без решения этих вопросов вряд ли возможно рассчитывать на получение вакцин против африканской чумы свиней и особенно на их профилактическую и противоэпизоотическую эффективность в общепринятом понимании.

Ключевые слова: обзор, Р. Ю. Монтгомери, африканская чума свиней, текущая пандемия, GAP-анализ

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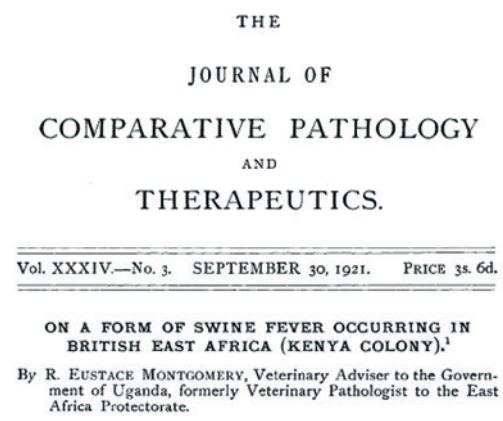
One hundred years ago Robert Eustace Montgomery (1880–1932) – distinguished veterinarian and pathologist, researcher of exotic diseases of the period of the first discoveries (Photo 1) – published results of the large-scale observations and experiments on African swine fever (Photo 2).

According to the Biographical Database of Southern African Science [1], R. Montgomery graduated at the Royal (Dick) Veterinary College in Edinburgh, Scotland, in 1903. After special duty in India and Canada, in 1907 he was sent by the Liverpool School of Tropical Medicine to investigate sleeping diseases (zoonosis, serious disease of humans and domestic animals) in Central Africa. The results of his investigation were presented in several papers published in the *Annals of Tropical Medicine* (1908–1909). In 1909 he was appointed as a pathologist to the East African Protectorate and in the same year participated to the Pan-African Veterinary Congress held in Pretoria on occasion of official opening of the Veterinary Research Institute in Onderstepoort, where he presented a report on "Trypanosomes and their transmission (Fly Disease) in relation to South Africa", published later in the *Proceeding of the Rhodesia Scientific Association*. He visited South Africa again in 1912 in connection with cattle immunization against East Coast fever. During the First World War (1914–1918) he served with the rank of Major in the East African Veterinary Corps. In 1917, R. Montgomery started the Veterinary Institute at Kabete (Kenya) and in 1918 he was appointed Director of Veterinary Research for the Union of South Africa. In 1920–1921, he was the first President of the South-African Veterinary Medical Association. From 1923 to 1926 R. Montgomery was a veterinary adviser to the governments of Kenya, Uganda, Tanganyika, and from 1930 – to the Colonial Office.

A truly fundamental R. Montgomery's contribution to veterinary science and practice is associated with the discovery of African swine fever (ASF) and the generalization of multi-year investigations of this newly discovered disease and some related phenomena, such as epizootology of exotic infections of wild animals, their role as reservoir, endemicity, that were non-trivial for the early XXth century. Current situation demonstrates that his contribution is truly epochal[1]. Detailed final report on the studies of African swine fever at the Nairobi Veterinary Laboratory (Kenya) started in 1900 was compiled by him in 1917 but it was published only four years later due to external reasons (war, change of jobs) [1, 3, 4].

[1] The second name of ASF is a *Montgomery disease* in the honor of R. Montgomery.

Photo 1. R. Eu. Montgomery [1]



THE

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AND

THERAPEUTICS.

Vol. XXXIV.—No. 3. SEPTEMBER 30, 1921. PRICE 3s. 6d.

ON A FORM OF SWINE FEVER OCCURRING IN BRITISH EAST AFRICA (KENYA COLONY).[1]

By R. EUSTACE MONTGOMERY, Veterinary Adviser to the Government of Uganda, formerly Veterinary Pathologist to the East Africa Protectorate.

Photo 2. Reprint of the historical publication on African swine fever [2]

In a large two-part paper [3], R. Montgomery gave the first description of signs of an unknown disease that emerged during the first attempts of rearing of pigs of European breeds imported from the metropolises by white settlers for home consumption or pork production. Cases of the disease, very similar to classical swine fever (CSF) well known in Europe, were officially registered on several farms in British East Africa but supposedly had a broader, epizootic, distribution.

Natural and experimental infection clinical picture and pathomorphology were described, infection natural reservoir and source (persistently and asymptomatically infected local warthogs – *Phacochoerus africanus*) were identified as well as the infectious agent was found to survive outside the animal body and in animal carcasses for a long time. Domestic pigs appeared to be fatally susceptible to parenteral infection with warthog blood but the infection was not transmitted by contact or by air that was interpreted as indicative of animal reservoir resistance and even then of low ASF contagiousness[2].

Experiments on cross-infection of CSF-immune pigs and passive protection from the infection with anti-CSF immune serum effectively and widely used for simultaneous vaccine-serum inoculations in pig industry at that time showed that the disease under study was new. Sera from wild pigs were also ineffective, i.e. fundamental fact of the lack of humoral protection against ASF became known even then. Attempted immunization with the heat-inactivated infectious material had no effect.

New infection was finally defined as an independent nosological form named as *East-African swine fever* in 1910. Further characterization of ASF, its causative virus, main principles of immunology and epizootology up to modern studies has improved the basic knowledge on the disease confirming the postulates laid down in the outstanding R. Montgomery paper [1, 3, 4].

In this context, current panzootia and resulting known stalemate undoubtedly require extraordinary solutions. Based on analysis of the data from PubMed[3] [5], worldwide ASF-associated publication activity highly intensified in recent years (Fig. 1) reflects both great scientific interest and scope of research activities.

A lot of international institutions and specially established associations with the World Organisation for Animal Health (WOAH) and Food and Agriculture Organization of the United Nations (FAO) as major players are involved in this common work. It should be noted that the following systems and organization are also highly engaged in the said activities: EMPRES (Emergency Prevention System for transboundary animal and plant pests and diseases, FAO), GF-TADs (Global Framework for the Progressive Control of Transboundary Animal Diseases, FAO), GARA (Global African Swine Fever Research Alliance, USA), Stop ASF (Public and private partnership programme, FAO/OIE), EFSA (European Food Safety Agency, EU), VACDIVA (EU Horizon 2020 Project on development of effective vaccine against ASF). Each organization combines and coordinates the research activities of several dozen up to more than one hundred and fifty participants (laboratories and institutions). The research effectiveness is discussed and evaluated in detail followed by development of agreed conclusions and solutions at regular scientific events and webinars[4]. Research outcomes are presented in a wide range of publications.

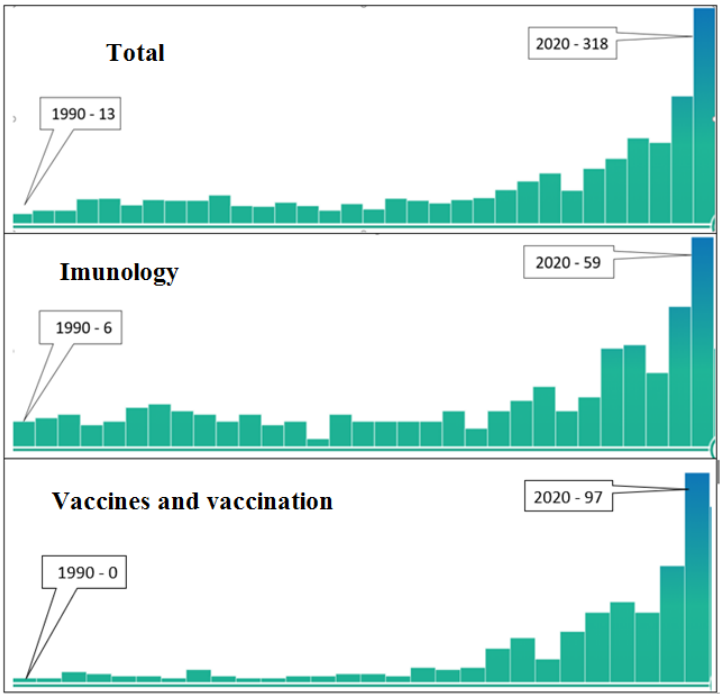

Fig. 1. Thirty-year dynamics of annual full-text publications on key ASF aspects (in call-outs) [5]

The EFSA Panel On Animal Health and Welfare (AHAW) activities and extended reporting and analytical publications in the *EFSA Journal* (for example, [6]) are of particular interest.

In particular, significant results were obtained during the Gap analysis[5] of management of short-term ASF risks for the disease control recently performed by a large team of reputable scientists and specialists in view of the aggravating disease situation in the participating European countries and published in the abovementioned periodical [5]. The timeliness of the said Gap analysis is obviously justified by rapidly increasing spontaneous large body of unsystematic publications that are still not associated with any significant anti-epizootic effectiveness.

Since the Gap analysis methodology is based on expert opinion of the persons whose interests are in any way related to the addressed task, respondents of the widest stakeholder range from pig farmers, hunters, veterinarians up to officers at high managerial level and all relevant officials and private professionals engaged in ASF control – national Veterinary Services and Ministries of Agriculture, European Veterinary Association, farmer's organizations, hunters' organizations, agribusiness, environment protection and management organizations, etc., taking into account different epizootogical status of the represented territories (first infected or endemic,

[2] This question is still debated. According to modern concepts, this type of pathogen-host relationship is defined as *persistent tolerant infection*.

[3] PubMed® is the largest data base of medicinal and biological publications at the US National Library of Medicine maintained by the National Centre for Biotechnology Information (USA), it contains more than 33 million citations of scientific literature (journals, books, other publications) [5].

[4] For example, recent public VACDIVA workshop (First International Workshop for the Pig Sector, October 1, 2021).

[5] Gap analysis is a method (or process) of the strategic analysis that compares actual result with what was expected for the identification of the challenging areas – suboptimal or missing strategies, structures, capabilities, processes, practices, technologies, skills, and recommends tools for the situation improvement and for meeting the goals. Unlike risk assessment focused on the future, the object of Gap analysis is the current state [7]. It is well known that recently Gap analysis has been actively used by the WOAH to improve the national veterinary service performance (PVS Pathway).

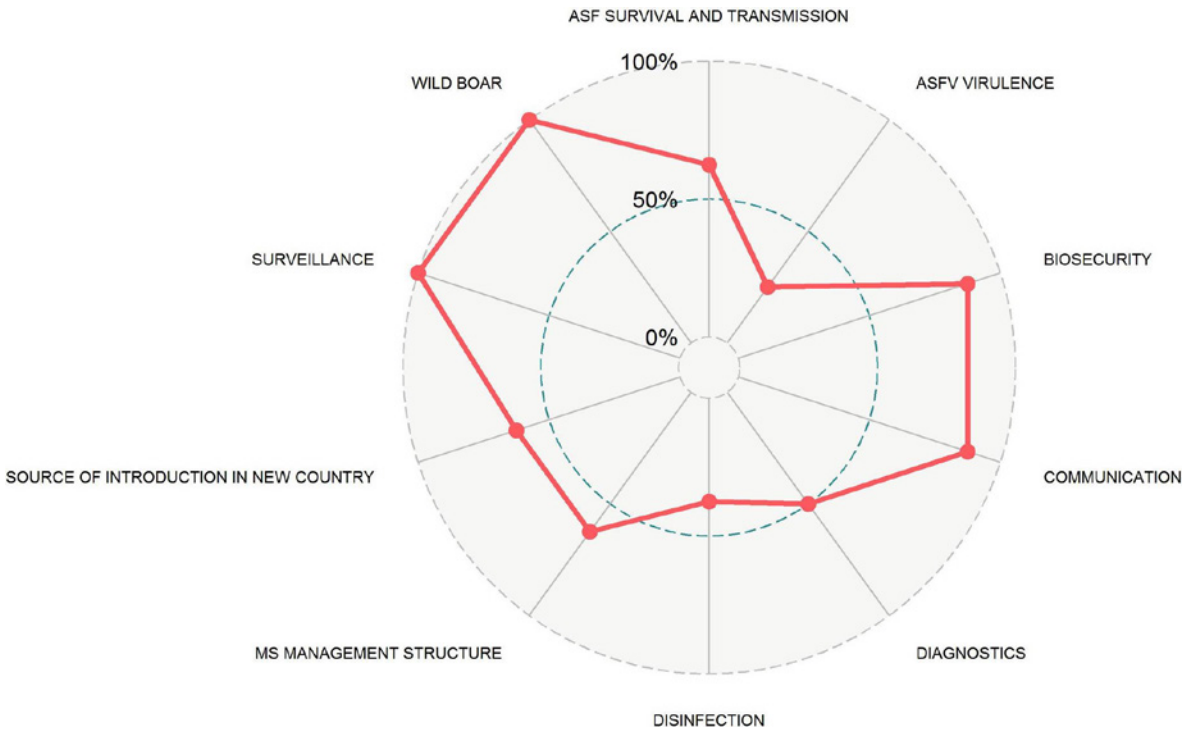

Fig. 2. Prioritization of currently important aspects identified by the Gap analysis, in percentage [6]

at infection risk, infection free) were involved to ensure its objectivity.

For an expert assessment of relevance based on a total analysis of the results published around the world, a number of "hot" issues covering the most currently important relevant scientific and practical areas in the context of an uncontrolled aggravation of the situation primarily in Europe were formulated. In particular, they included (*i*) role of survived animals in further ASF spread, (*ii*) virus virulence and possibility for live vaccine development, (*iii*) biosecurity in the broad sense of controlling infection spread risks, (*iv*) intersectoral communications for maximum coordination of all stakeholders' actions, information sharing, training, (*v*) sensitivity of diagnostic tests, non-invasive methods for wild boar, (*vi*) disinfection of various objects, destruction of wild boar carcasses, (*vii*) contribution of governmental bodies to international cooperation and funding, (*viii*) role of import, migration, pathways, objects in the infection introduction to the infection-free countries, (*ix*) passive surveillance in wildlife, border control, zoning for trading, early infection detection, (*x*) ecology, epizootology, control of wild boar populations. The Gap analysis results were summarized and presented in the form of spider graph (Fig. 2).

The conclusions and recommendations arising from this prioritization indicate the main point: the most popular trivial and conventional directions in modern ASF research, such as diagnostics (availability of faultless polymerase chain reaction), disinfection (everything was done a long ago), are not currently significant, and numerous publications (see Fig. 1) show that the main gap associated with the virus virulence studies (endless genotyping) is the unsuitability of the proposed attenuated virus variants as vaccines, according to generally accepted immunology and protectivity concepts. The main challenging gaps requiring primary attention were identified in four out of ten categories.

The crucial gaps in relation to *wild boar* were both organizational ones – necessity of harmonized wild boar population density estimation and ASF reporting in the wild, determination of the methods for reducing the absolute number of wild boar – and scientific ones – elucidation of the mechanisms for ASF spread and ASF virus persistence in the wild, possibility and importance of direct-contact infection transmission in wild boar taking into account their behavior.

In relation to *ASF survival and transmission*, it was shown that process management requires better knowledge and understanding of the insect vector role in biological and mechanical transmission of the infection, role of contaminated abiotic environmental factors and feed, potential risks associated with production, processing, transportation, storage of forage materials, different beddings, household items, porcine products and with personnel.

In relation to *biosecurity*, the important identified gaps were as follows: lack of effective measures for prevention of transboundary ASF introduction in the regions and holdings with different husbandry systems, for prevention of the infection transmission between wild boar and domestic pigs as well as potential risks of backyard farm involvement. Other gaps were considered serious: low public awareness of ASF, and the fact that social and economic situation, rural community lifestyle, as well as traditional agricultural practices were not appropriately taken into account.

The following was considered of high priority for the *surveillance* aimed at mitigation of risks of ASF introduction to the disease-free countries/regions: enhancement of border control of people, transportation vehicle, commodity movements, passive surveillance for and early detection of

dead animals (primarily, wild boar), methods for the agent detection (forage materials, items after decontamination), sensitive and rapid pen-side diagnostic kits to be used in the field, non-invasive tests for wild boar [6].

A common disadvantage of the said Gap analysis is, of course, the absence of the questions on ASF immunology addressed to the well-known numerous gaps in the questionnaire, while all efforts are focused on identification of virulence genes for their removal and virus attenuation. According to the recent publications with the participation of J. M. Sánchez-Vizcaíno [8], despite of a wide information flow, knowledge about immune defense mechanisms is very scarce. Such topic segregation can be attributed for the peculiar local interests of the Western European community in the current context.

However, in another comprehensive Gap analysis with a similar set of questions carried out on the GARA initiative (USA) [2], immunity-associated challenges were also not adequately addressed. The following clearly chronic gaps remain beyond the routine research: virus interaction with macrophages and, in general, a role of these unique cells in pathogenesis and immunity, virus-induced intracellular processes and virus reproduction, hemadsorption as antigenic modulation of host cell, hemadsorption antigen, serotyping hemadsorption-inhibition test, other *in vitro* phenotypic close-to-reality signs correlating to virulence and immunity, namely, intrapopulation virus heterogeneity, lack of protective humoral response of the immunity system (trivial virus neutralization), culture models for direct *in vitro* testing of protective antigen expression and phenomenology of cellular and intercellular protective reactions (interactions of antibodies with virus particles, $CD8^+$ T-lymphocytes-killers and other cytotoxic effectors with infected target cells, immune cytolysis morphology), effector potential of T-cell-mediated immunity.

It is hardly possible to expect development of anti-ASF vaccines and particularly vaccines capable of preventing and effectively protecting against ASF epizooty according to general understanding without addressing these issues, especially without determination and comprehensive characterization of the immune response effector phase using modern research methods, focusing only on acquired resistance to challenge (i.e. at the animal level). At the same time, the answers to many of the above questions were obtained earlier during the research carried out by the Biochemistry and Immunology Laboratories of the Federal Research Centre for Virology and Microbiology (Pokrov, Russia) and widely covered in the national scientific literature [9, 10].

It is noteworthy that the American Gap analysis, probably for the first time in relation to ASF panzootia control, raises the question about the reasonability of total destruction of suspected susceptible population including emergency slaughter of tens of thousands of pigs that results in significant financial losses, provokes all forms of owners' resistance and is ethically challengeable. Hence, alternative solutions to this socio-economic problem addressing the general public requirements without prejudice to effectiveness of anti-epizootic measures are required [2].

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Density of wild boar population and spread of African swine fever in the Russian Federation

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SUMMARY

African swine fever (ASF) is a transboundary viral disease affecting all species of the *Suidae* family. It greatly undermines global pig industry and causes a significant damage to the ecology of the wild boar (*Sus scrofa*) which is a natural reservoir of the ASF virus and is an intermediate link in the epizootic process. Depopulation of wild boar is one of the measures taken to prevent spread of ASF in the Russian Federation. A threshold density of the wild boar population of 0.25 head/1000 ha (0.025 head/km^2), according to the National Plan on the ASF Eradication in the Russian Federation, was achieved by 2020 in many RF Subjects. However, further analysis of the ASF epizootic situation shows that the measure has failed to eradicate the infection completely. A regression analysis showed statistically significant positive relationship between recurrent ASF outbreaks in the wild boar population and its density in a number of model subjects (N = 6). At the same time, there is no such dependence in other model subjects (N = 3), and ASF outbreaks were recorded in wild boars at a density significantly lower than the recommended threshold value. A review of foreign and national scientific publications has shown that such control methods as depopulation is just one part of the whole set of measures taken to eradicate ASF in the wild. The measure is effective only when 70–80% of animals are culled in a short time, which is practically impossible due to the high costs and some peculiarities of the population control and depopulation process. Based on the results obtained, it can be concluded that a decrease in the number of wild boars does not guarantee to stop further spread of infection in the Russian Federation and it should be considered as just a part of the whole set of anti-epizootic measures taken together with other anti-epizootic measures to eliminate and prevent ASF.

Keywords: African swine fever, density of wild boar population, depopulation, logistic regression, elimination strategy

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Плотность популяции дикого кабана и распространение африканской чумы свиней в Российской Федерации

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РЕЗЮМЕ

Такое вирусное трансграничное заболевание всех представителей семейства свиньи (*Suidae*), как африканская чума свиней, наносит колоссальный ущерб не только мировой свиноводческой отрасли, но и экологии кабана (*Sus scrofa*) – животного, являющегося природным резервуаром вируса и участником эпизоотического процесса. Одной из мер по предотвращению распространения африканской чумы свиней на территории Российской Федерации является депопуляция дикого кабана. Рекомендуемое «Планом действий по предотвращению заноса на территорию Российской Федерации африканской чумы свиней и ее распространения» значение плотности популяции кабана в 0,25 особи/1000 га (0,025 особи/км^2) для многих субъектов страны было достигнуто к 2020 г., но, как показывает анализ эпизоотической ситуации по африканской чуме свиней, данная мера не привела к полному искоренению инфекции в Российской Федерации. Регрессионный анализ показал, что в ряде модельных субъектов (N = 6) прослеживается статистически значимая положительная взаимосвязь между наличием повторяющихся вспышек африканской чумы свиней в популяции дикого кабана и ее плотности. В то же время в других модельных субъектах (N = 3) такая зависимость отсутствует, а вспышки африканской чумы свиней регистрировались среди диких кабанов при плотности, существенно меньшей рекомендуемого значения. Обзор зарубежной и отечественной научной литературы показал, что применение методов контроля численности кабанов, таких как депопуляция, является лишь частью комплекса мер по искоренению африканской чумы свиней в дикой природе и эффективно лишь при изъятии 70–80% особей в короткие сроки, что практически нереализуемо в силу высоких экономических затрат и нюансов применения методов контроля и сокращения популяции. Исходя из полученных результатов, можно сделать вывод, что снижение численности дикого кабана не является гарантией прекращения дальнейшего распространения инфекции на территории Российской Федерации и должно́ рассматриваться в составе комплекса мер, направленных на ликвидацию и предупреждение заноса африканской чумы свиней, наряду с другими противоэпизоотическими мероприятиями.

Ключевые слова: африканская чума свиней, плотность популяции дикого кабана, депопуляция, логистическая регрессия, стратегия ликвидации

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INTRODUCTION

African swine fever (ASF) is a viral, transboundary disease affecting all species of the *Suidae* family and causing both an enormous damage to the national pig industry and to the ecology of the wild boar. Affected and convalescent pigs and wild boar and as well as those ones shedding the virus without clinical signs during an incubation period are the source of the pathogen. Extensive research into the role of the wild boar in ASF spread has revealed that this animal is an important, but not the key factor in the disease spread in the Russian Federation [1–3]. The wild boar is known to support ASF enzootoicity in the territory [4–6]. The ASF outbreaks recorded in the wild in the Russian Federation throughout the whole period of the disease control are still mostly sporadic. Environmental risk factors preserve and maintain virulence of ASF virus in the environment and thereby complicate the disease elimination [7]. Mostly, the infection sources remain unknown due to both peculiarities of backyard pig farming and hunting farms, characterized by uncontrolled movement of animals, migration of wild boar, transportation of pig products and hunting trophies [4, 7, 8]. While lack of biosafety policies on pig farms is considered the main factor in the disease spread [9–13], the presence of wild boar in the ecosystem plays an important role in ASF transmission to the domestic pigs, as recognized by many countries [9, 14, 15]. Circulation of ASF virus in the wild boar population is typical for the Russian Federation and some of its subjects [16]. Recently, such a mechanism of ASF epizootics has been clearly observed in the Far East [17–19].

Currently, discussions are under way as to the relationship between ASF spread and density of the wild boar population. Taking into account experience of the European countries, there is a strong dependence of the virus transmission on density of the wild boar population; however, this dependence is not always observed [20]. Due to peculiarities of ASF epizootic process, this trend mainly depends on:

– network structure and social interactions in the most susceptible wild boar population and between age and gender groups;

– unclear pattern of the animal-to-animal virus transmission and post-mortem virus stability in dead boar, depending on the environmental conditions (for example, air temperature).

Studies in Poland, Germany and Italy, T. Podgórski et al. [21] reveal that the frequency of contacts within social groups was 17 times higher than between animals from different groups. These interactions suggest a mature metapopulation in which intra-group transmission happens faster, and the spread of infection between groups is limited and prolonged. The authors also found that young wild boar interact with each other more frequently in the population and such contacts can speed up the infection transmission. A wild boar population management strategy that affects the social-spatial structure of the population, for example, extra feeding, may reduce the time of virus transmission, because the likelihood of contacts between different groups increases.

Thus, ASF outbreaks reported in Poland from 2014 until mid-2016 may have resulted from a higher density of wild boar population (1–4 boar/km^2) in the east and a lower density in the west (< 0.4 boar/km^2). Z. Pejsak et al. [22] assumed that a density of more than two animals per square kilometer is required in order to ensure stable circulation of the virus among wild boar in Poland.

The theory of threshold density values does not give clear answers on principles of ASF virus spread, virus persistence in the wild boar population and transmission of the pathogen to other susceptible populations, including domestic pigs. Model approaches are based on such key conditions as homogeneous and random interaction between the sick and healthy animals, which is unlikely to really happen in the wild. Beside the density of the wild boar population, the virus transmission dynamics in the population can be influenced by such factors as post-mortal ASF virus stability in the dead wild boar, the population social structure, mechanical carriers, and etc. Therefore, the threshold values of the boar density will not necessarily reflect the possibility of transmission in a particular area. In addition, due to their social behaviour, animals can group together even in those areas and territories where population density is low, thus, zones with more wild boar will appear creating conditions for new ASF outbreaks.

Studies of the wild boar population ecology conducted within ENETWILD project [13, 23] and EFSA [24] have revealed that field observations are the only available alternative approach to study population density thresholds in the context of ASF prevention and control.

Strategically important disease control measures include, *inter alia*, wild boar depopulation, i.e. reduction of wild boar density to a certain threshold at which intrapopulation virus transmission will stop or significantly slow down due to a decrease in the reproduction coefficient [25–27].

Based on the current analysis of ASF epizootic situation in the Russian Federation, it can be said that the disease has spread both in wild boar and in domestic pigs almost throughout the whole territory, including even those regions where, as stated, density of the boar population is very low. Therefore, the purpose of this study is to examine the relationship between ASF outbreaks in wild boar and the wild boar population density in the Russian Federation.

In order to achieve the purpose, the following objectives have been set:

1) to conduct a retrospective analysis of the ASF situation in wild boar in the RF subjects and to determine those model and ASF-enzootic RF subjects, where wild boar populations have been continuously affected by the disease for several years;

2) to collect data and analyze relationship between dynamics of the wild boar population density in the model Subjects of the Russian Federation and the number of ASF recurrent outbreaks;

3) to determine whether there is a statistically significant relationship between occurring ASF outbreaks and changes in the density of wild boar population resulting from hunting activities, as well as depopulation, which is an important measure for ASF elimination;

4) to review scientific literature on the wild boar ecology in the ASF-affected environment with the purpose to systematize the methods applied to reduce ASF virus circulation in the population.

MATERIALS AND METHODS

Based on the use of PRISMA (http://www.prisma-statement.org/PRISMAStatement/PRISMAStatement.aspx) statement for systematic reviews and meta-analyses [28], a literature search was conducted in Web of Science, PubMed, Scopus and Google Scholar databases to find relevant information on the methods and tools potentially applied to ensure freedom of the wild boar population from ASF. The search query included the following keywords: African swine fever, population density of wild boar, depopulation, logistic regression, elimination strategy, while no publication date filter was applied. A literature search was also carried out in RSCI bibliographic database (Russian Science Citation Index) using Science-Index. For this purpose, we firstly reviewed headings and summaries, then analyzed full texts of the papers identified as relevant.

Model regions. Based on a retrospective epizootological analysis of the ASF epizootic situation, the following subjects of the Russian Federation were selected as model regions, where ASF outbreaks in wild boar recurred from 2013 to 2021, and for them data were available on long-term changes in the number of animals and population density during the epizooty (at the municipal level): the Vladimir, Yaroslavl, Ryazan, Nizhny Novgorod, Samara, Saratov and Amur Oblasts, as well as the Khabarovsk and Primorsky Krais.

Within this study, an outbreak is defined as an occurrence of one or more ASF cases in an epizootological unit (municipal district of the Russian Federation). At the same time, a case is defined as an individual animal infected with the pathogen, either with clinical signs or without them[1].

Data on ASF registration in the wild boar population are taken from the official reports of the Federal State-Financed Institution FGBI "Veterinary Center" (Moscow)[2].

Data on the size and density of wild boar population in municipal districts are taken from the regional websites of the Ministry of Natural Resources and Hunting Committees[3].

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[3] The Ministry of Natural Resources of Russia. Available at: https://www.mnr.gov.ru.

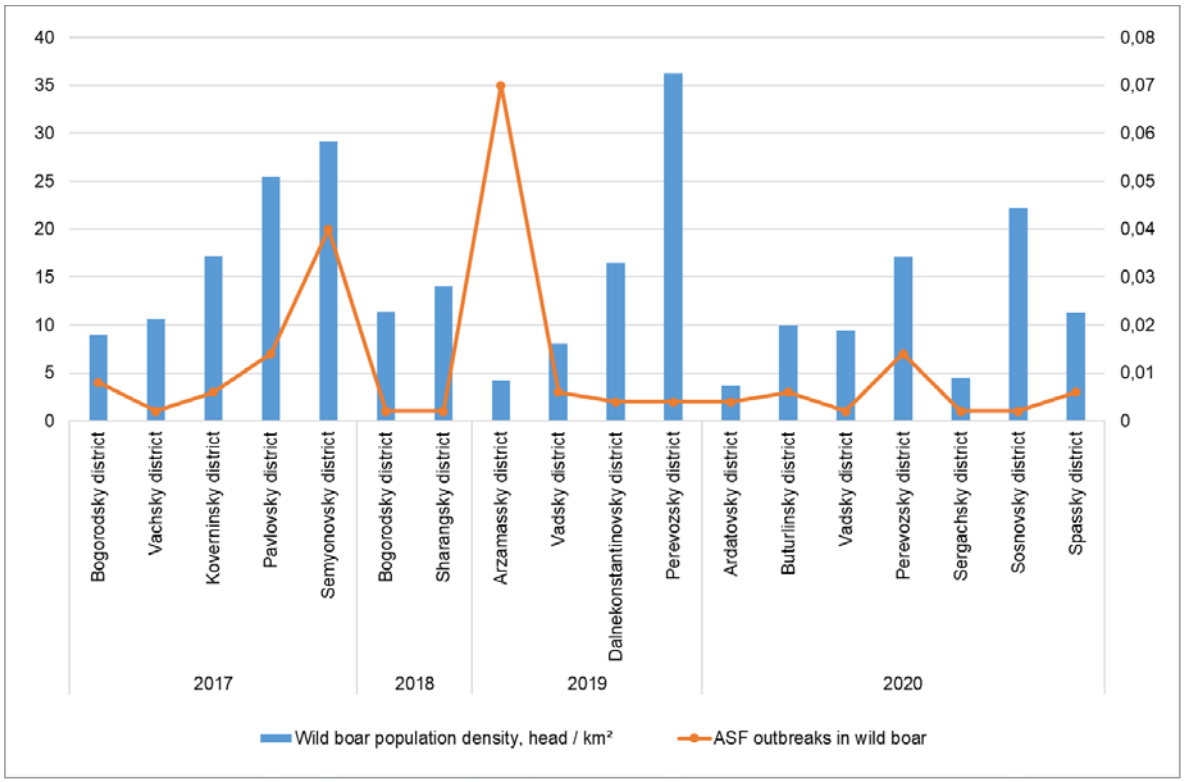

Fig. 1. Frequency of ASF outbreaks in the wild boar population of the Nizhny Novgorod Oblast (2017–2020)

Epizootological information on ASF outbreaks and data on size and density of the wild boar population covered the period from 2013 to 2021.

We used generalized linear logistic regression (GLLR) method to model relationship between ASF outbreaks registered in wild boar and the dynamics of the population density. The method examines the relationship between a dichotomous variable ("yes/no") and one or more explanatory factors [29–31]. For the purpose of this study, the explanatory variable refers to the presence/absence of registered ASF outbreaks in the wild boar population in a particular municipal district, and the explanatory factor refers to the population density of wild boar in the municipal district for the corresponding year. The significance of the explanatory variable was assessed using the Student's *t*-test (statistical criterion $p_t < 0.05$ indicates the significance of the variable as an explanatory factor). The overall statistical significance of the models was assessed using Hosmer – Lemeshow goodness-of-fit test that determines the ratio between the numbers of expected and observed events in subgroups of the model population. Statistical reliability of this test at $p_{hs} > 0.05$ demonstrates sufficient predictive ability of the model.

In addition to model significance and reliability, we calculated an odds ratio (OR) for a positive outcome in each subject and compared this coefficient based on the explanatory variable contained in the model (wild boar population density).

Logistic regression in R programming language[4] was used to model the relationship between ASF outbreaks and the wild boar population density at the municipal level.

Data on ASF outbreaks and wild boar population density in model regions were mapped with the help of ArcGIS for Desktop 10.8.1 geographic information system (ESRI, Redlands, California, the USA).

RESULTS

Retrospective epizootological analysis. Scientific literature screening focused on techniques that ensure freedom of wild boar population from ASF helped to select 45 reviews from international scientific citation databases and 40 scientific papers from the RSCI database that meet the search criteria. Summarizing results reported in these studies, we described in section "Discussion" different opinions on the role of wild boar population density in ASF spread and its persistence in the population.

A retrospective epizootological analysis showed that, from 2013 to 2021 according to the FGBI "Veterinary Center", totally 2,036 ASF outbreaks occurred in the model regions, of which 1,181 occurred in the population of domestic pigs and 855 in wild boar.

The highest total numbers of ASF outbreaks in wild boar was observed in the Saratov (128), Samara (95), Volgograd (84) Oblasts, the Primorsky Krai (80), Amur (69), Voronezh (52), Moscow (52) Oblasts and the Khabarovsk Krai (47).

An epizootological analysis of the ASF situation in the model regions showed stationary nature of the disease outbreaks in wild boar. The stationary nature of outbreaks is mainly typical for endemic diseases, characterized by the ability of the causative agent to exist long in certain

[4] R-4.1.1 for Windows. Available at: https://cran.r-project.org/bin/windows/base.

territories among wild animals permanently living there. Outbreaks of the disease may recur at various intervals because conditions for their recurrence exist. Frequency of ASF outbreaks in wild boar in the same areas of the model regions makes it possible to define them as stationary, for example, in the Nizhny Novgorod Oblast (Fig. 1).

Modeling relationship between ASF outbreaks and population density of wild boar. Modeling relationship between ASF outbreaks and population density of wild boar was carried out at the municipal level:

1) for all the selected model subjects in general;
2) for every subject individually.

General modeling for all model subjects showed both statistical insignificance of the boar population density as an explanatory factor ($p_t = 0.42$) and the unsatisfactory result of the Hosmer – Lemeshow test ($p_{hs} < 0.01$) revealed poor explanatory ability of the model. This allows us to conclude that it is impossible to establish in general a clear correspondence between the density of wild boar populations and repeated ASF outbreaks within the model region (Table).

At the same time, modeling for some subjects of the Russian Federation showed that most subjects (70%), 6 out of 9 (the Khabarovsk Krai, the Primorsky Krai, the Amur Oblast, the Vladimir Oblast, the Ryazan Oblast, the Saratov Oblast) demonstrate statistically significant ($p_t < 0.05$) positive dependence of ASF outbreaks on density of the wild boar population (Fig. 2).

The results obtained for odds ratio indicate that the dependence of ASF outbreaks on density of the wild boar population may be observed more in the Vladimir, Ryazan and Saratov Oblasts, as well as in the Khabarovsk and Primorsky Krais. At the same time, the greatest statistical reliability of dependence of the phenomenon on the explanatory factor (population density) was observed for the Vladimir Oblast, Primorsky and Khabarovsk Krais. That is, it can be concluded that the higher odds ratio

Table
Modeling dependence of ASF outbreaks on the density of wild boar population in the Russian Federation (2013–2021)

Subject of the Russian Federation	ASF outbreaks registered in (years)	Number of ASF outbreaks/number of cases in the wild boar population	Odds ratio (OR)	p_t-value GLLR models
Vladimir Oblast	2013 2015 2016 2017 2018	2/13 1/12 17/38 8/41 1/1	6.58×10^6	0.002**
Yaroslavl Oblast	2013 2015 2019 2021	22/52 2/61 1/4 4/4	47.94	0.442
Ryazan Oblast	2015 2016	22/52 44/341	12,456.52	0.018*
Nizhny Novgorod Oblast	2016 2017 2018 2019 2020 2021	1/5 20/35 2/2 5/42 9/18 1/1	2.61	0.326
Samara Oblast	2020 2021	60/163 2/9	7.92	0.116
Saratov Oblast	2015 2016 2017 2021	4/10 8/26 5/10 1/7	121.75	0.009**
Amur Oblast	2019 2020 2021	1/18 8/32 1/1	113.07	0.05*
Primorsky Krai	2019 2020 2021	20/41 42/128 10/20	81.31	0.005**
Khabarovsk Krai	2019 2020 2021	6/7 18/29 3/3	824.68	0.004**
Model Subjects	2013–2021	351/916	1.15	0.420

GLLR is a generalized linear logistic regression model;
OR – odds ratio (1/0), with a value of $p_t < 0.05 - n^*$, $p_t < 0.001 - n^{**}$.

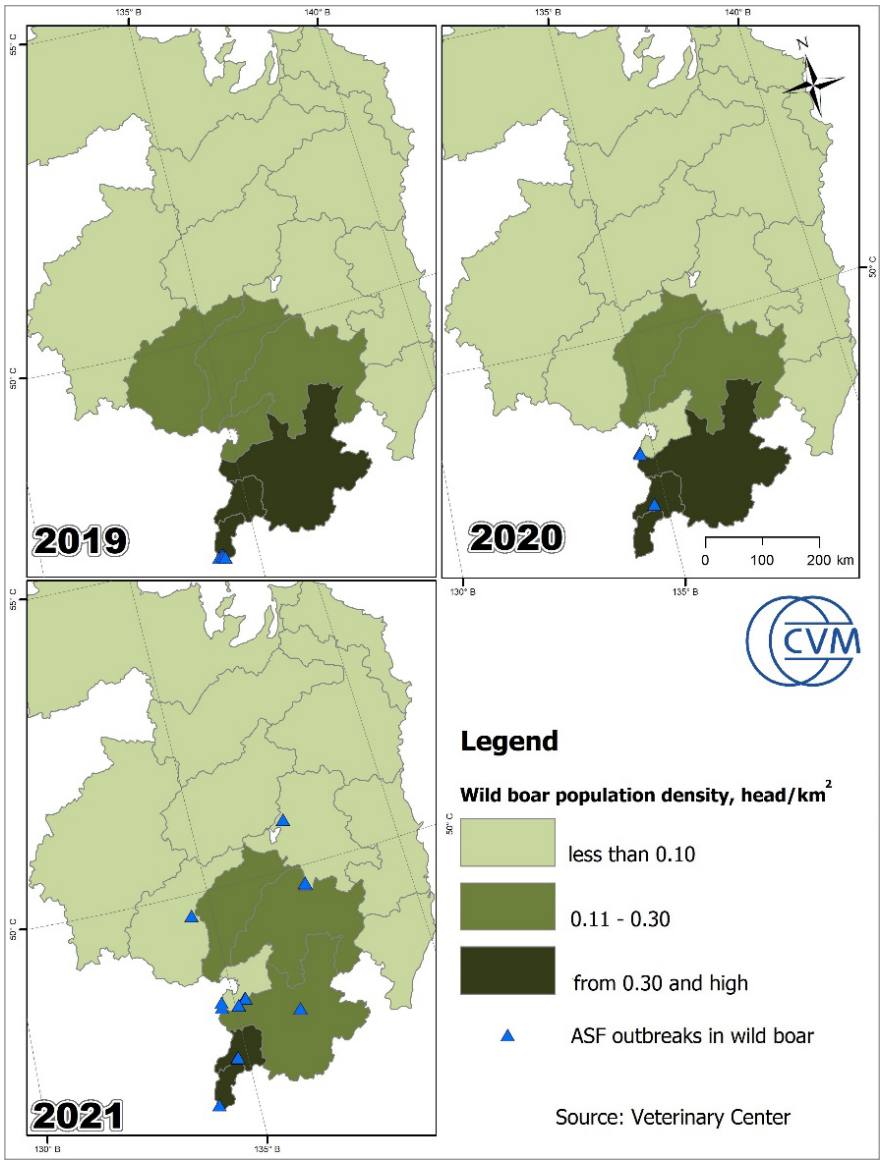

Fig. 2. Changes in the ASF epizootic situation in the Khabarovsk Krai related to the density of the wild boar population (2019–2021)

is (when OR > 1), the higher the chances are to identify the risk factor and the dependence of the recorded ASF outbreaks on the density of the wild boar population.

However, three out of the nine model subjects (the Nizhny Novgorod, Samara and Yaroslavl Oblasts), demonstrated no such dependence. In these model subjects, ASF outbreaks were reported even in the areas where the wild boar population density is significantly lower than the recommended value of 0.25 head/1000 ha (0.025 head/km^2), as approved by Order of the Government of the Russian Federation dated 30.09.2016 No. 2048-r (as amended on 04.02.2021) "Action Plan to prevent introduction of African swine fever into the Russian Federation and its spread in the country"[5].

[5] Order of the Government of the Russian Federation dated 30.09.2016 No. 2048-r (as amended on 04.02.2021) "On approval of the action plan to prevent introduction of African swine fever into the Russian Federation and its spread in the country". ConsultantPlus. Available at: http://www.consultant.ru/document/cons_doc_LAW_205372.

Figure 3 shows as an example the dependence of ASF outbreaks in wild boar on population density from 2017 to 2020 in the Nizhny Novgorod Oblast. In all the cases reviewed, the models demonstrated a satisfactory result of Hosmer – Lemeshow test ($p_{hs} > 0.05$), suggesting sufficient predictive ability of the models.

DISCUSSION

The epizootic situation on ASF is currently tense in the subjects of the Russian Federation, due to outbreaks reported both in domestic pigs and wild boar. Despite the measures taken to prevent ASF spread in the wild, ASF introduction in the wild boar population is still reported in previously disease-free areas. The recorded ASF outbreaks and the decreasing trend of wild boar population density in the tested model subjects indirectly confirm the assumption that wild boar play some role, but not a major one in ASF spread. The following measures are regularly taken to control ASF in the areas previously affected by

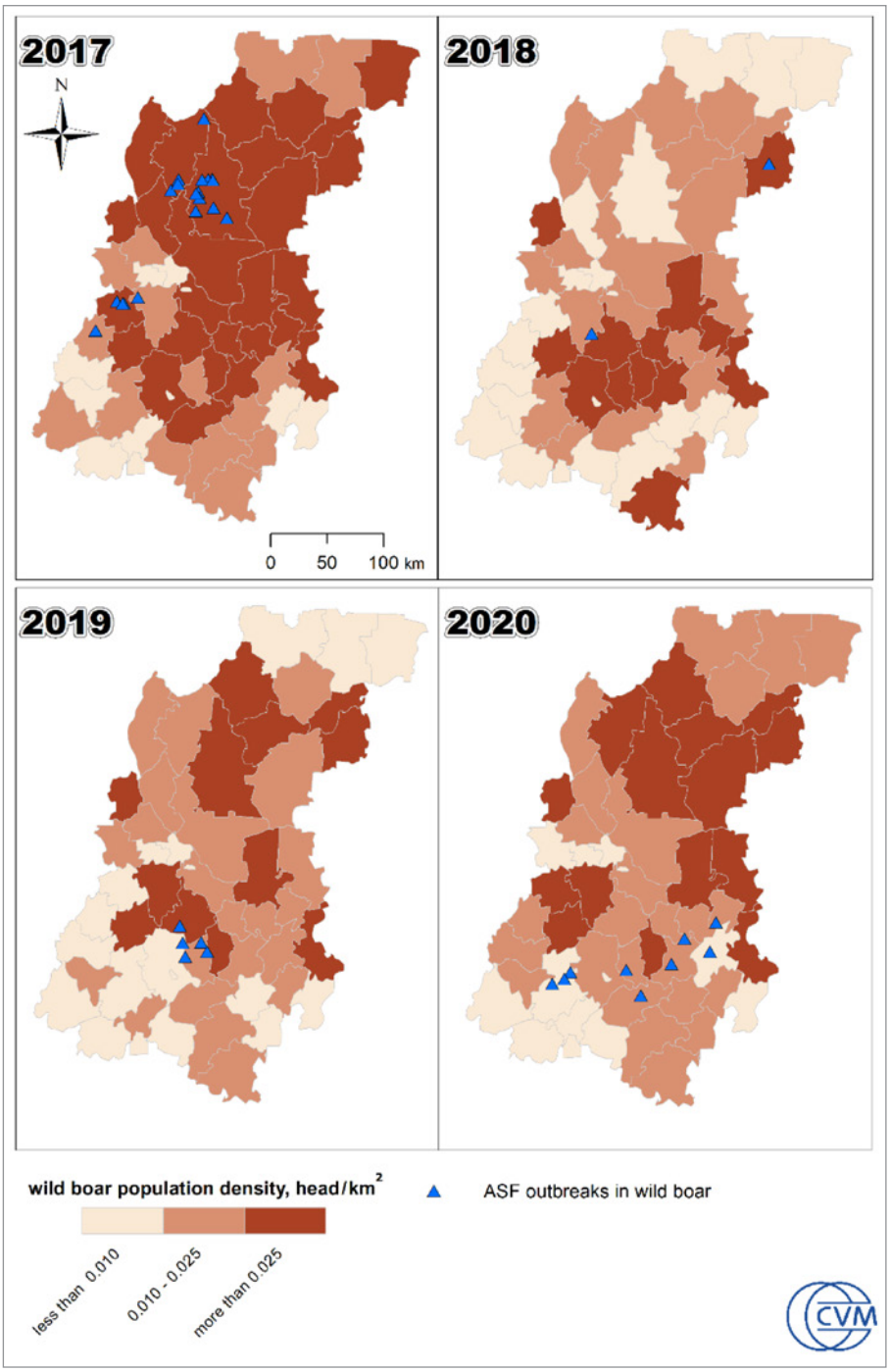

Fig. 3. Changes in the ASF epizootic situation in the Nizhny Novgorod Oblast related to the density of the wild boar population (2017–2020)

the disease: the wild boar population reduction, targeted hunting of female wild boar and removal of dead carcasses. These measures effectively reduce the risk of infection.

Although some researchers suggest a threshold density of wild boar population, which can stop ASF spread in certain areas [21, 22, 26, 32], other authors believe that even if the value is reached, there is no guarantee that the epizootic chain will break [11, 20, 24, 33]. The current national legislation on prevention and control of ASF provides for animal population reduction in ASF-affected territories to the recommended value of 0.25 head/1000 ha, which can be achieved by intensive depopulation of wild boar in the tested regions.

Some researchers have shown that it is currently impossible to establish a threshold density for the wild boar population that can be considered critical to maintain the virus in the environment and keep its spread. Based on the analysis of domestic and foreign literature, various strategies for managing wild boar at certain stages of the ASF epizootic scenario are proposed [34–36]. Preventive measures, taken to depopulate and stabilize the wild boar population before ASF introduction, will help both to minimize

the likelihood of infection in the population and to reduce the costs and efforts required for potential emergency actions aimed at the disease eradication (lowering costs of searching for dead carcasses) [37–39]. Passive surveillance is the most effective method for early detection of ASF in the disease-free territories (search, safe removal and destruction of dead boar). Following ASF introduction into a particular region, no measures shall be taken in a short while in relation to wild boar populations (for example, a ban shall be imposed on hunting of all species, no crops shall be harvested to ensure food and shelter in the affected area), and only healthy wild boar population can be sharply reduced in ASF-free areas [26, 40]. Following a decline of ASF epizooty, when confirmed by passive epizootological surveillance, active population management should be applied. The positive trend, detected in the dependence of ASF outbreaks in wild boar in some RF model subjects, suggests there is a local-spatial effect of the wild boar density on ASF spread.

In general, considering dependence of ASF outbreaks on the population density for all the selected model subjects, no positive trend was observed, however, in some areas a regression analysis revealed a positive relationship. As for depopulation as a strategic measure to contain ASF, it can be assumed that its large-scale use can backfire and result in new outbreaks due to an increase in the average radius of the wild boar habitat [41].

This fact allows us to make a conclusion that the wild boar depopulation is a necessary strategic measure for ASF control and eradication, but only in certain disease-free areas bordering on the infected ones. In our opinion, an effective strategy to eliminate and prevent ASF spread in the wild should be based on the following principles:

– to ensure regular passive monitoring of ASF in the wild;

– to conduct mathematical and geographical modeling in order to establish the relationship between ASF outbreaks and wild boar population characteristics (density, structure);

– to control wild boar numbers and strictly comply with biosafety rules while hunting and dealing with dead carcasses;

– to isolate affected territories (recent studies have confirmed that wild boar demonstrate the same ASF pattern as the domestic pigs, i.e. the disease is acute, which reduces their role in the spread of infection);

– if ASF is introduced into a previously disease-free region, it is recommended to completely stop drive hunting, not to feed wild boar and, in general, not to take any actions to regulate the population size;

– to prevent further spread across the territory, number of wild boar can be significantly reduced in the areas adjacent to the affected areas before the disease introduction.

Incompleteness of data on population density provided on the municipal level in all the RF subjects is a major barrier to establishing dependence of emerging ASF outbreaks on the wild boar population density. As the data required become available, we will continue to fill in the data gap, in order to find the answer to the question, whether emerging ASF outbreaks depend on the density of wild boar population, and we will extrapolate the results obtained to the whole territory of the Russian Federation.

CONCLUSION

Statistical analysis has shown that there is no strong dependence of ASF outbreaks on the population density of wild boar in the model regions, although such dependence exists for a number of subjects of the Russian Federation. The obtained results suggest that reduction of wild boar population to the recommended density threshold does not prevent further spread of ASF and should be considered as one of the options in the set of measures together with the use of fences, suspension of feeding of wild boar and ban on drive hunting. Depopulation can be applied only in disease-free areas adjacent to the affected subjects (districts).

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Flow cytometry sorting of cells infected with African swine fever virus

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SUMMARY

The African swine fever panzootic is continuing to spread, and the number of affected countries and material losses are increasing. In particular, India, Papua New Guinea, Malaysia, Greece and Bhutan joined the list of ASF infected countries in 2020–2021. The disease control is hindered by the lack of commercially available and effective vaccines, which, in its turn, is attributable to the insufficient knowledge of ASF pathogenesis and immune defense against the disease. The use of attenuated virus variants enables a thorough investigation of the factors influencing the virulence of African swine fever virus and the immune response to it. This involves the use of naturally attenuated virus variants, as well as of the variants attenuated by a long-term passaging of the virus in cell cultures. However, virulence heterogeneity characteristic of the ASF virus population, necessitates the additional selection of infected cells for the virus cloning. Conventional culture-based techniques for virus particle cloning are rather time- and labour-consuming; it is therefore appropriate to use flow cytometry cell sorting for the selection and cloning of virus infected cells with a view of selecting homologous virus lineages. The paper presents the results of sorting of African green monkey kidney cells (CV-1) and porcine bone marrow cells infected with African swine fever virus; the cells were sorted into the 96-well culture plates using a MoFlo Astrios EQ cell sorter in order to isolate a population of the virus originating from one infected cell. After the single cell sorting of the infected cell cultures into the 96-well plates, ASF positive cell detection rates in the plate wells were 30% for porcine bone marrow cells and 20% for CV-1.

Keywords: African swine fever, cytometry, cell sorting, cell culture

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Сортировка клеток, инфицированных вирусом африканской чумы свиней, методом проточной цитометрии

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РЕЗЮМЕ

Панзоотия африканской чумы свиней продолжает свое распространение, а число пораженных стран и материальные потери увеличиваются. Так, в 2020–2021 гг. к перечню неблагополучных по африканской чуме свиней стран добавились Индия, Папуа – Новая Гвинея, Малайзия, Греция и Бутан. Борьбу с заболеванием затрудняет отсутствие коммерчески доступных и эффективных вакцин, что, в свою очередь, обуславливается недостатком знаний о патогенезе и иммунной защите при африканской чуме свиней. Детальное изучение факторов, влияющих на вирулентность вируса африканской чумы свиней и вызываемого им иммунного ответа, становится возможным при использовании его аттенуированных вариантов. Для этого применяют как естественно аттенуированные варианты вируса, так и варианты, аттенуированные в ходе длительного пассирования вируса на культурах клеток. Однако гетерогенность по признаку вирулентности, свойственная популяции вируса африканской чумы свиней, требует проведения дополнительного отбора инфицированных клеток с целью клонирования вируса. Классические культуральные методы клонирования вирусных частиц достаточно длительны и трудоемки, поэтому для отбора и клонирования инфицированных вирусом клеток с целью получения гомологичных вирусных линий целесообразно использовать сортировку клеток методом проточной цитометрии. В данной работе показаны результаты сортировки зараженных вирусом африканской чумы свиней клеток почки африканской зеленой мартышки CV-1 и костного мозга свиньи с помощью клеточного сортера MoFlo Astrios EQ в 96-луночные культуральные планшеты с целью получения популяции вируса, происходящего из одной зараженной клетки. После проведения сортировки инфицированных культур клеток по одной клетке в лунки 96-луночных планшетов частота обнаружения положительных на африканскую чуму свиней клеток в лунках составила 30% для клеток костного мозга свиньи и 20% – для CV-1.

Ключевые слова: африканская чума свиней, цитометрия, сортировка клеток, культура клеток

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INTRODUCTION

African swine fever (ASF) is a contagious septic disease that affects both domestic pigs (including miniature ones) and wild boar. Susceptible animals can develop an acute, subacute, chronic or asymptomatic form of the disease [1, 2]. The nature of the disease manifestations depends on the biological properties of the causative virus, as well as on the individual characteristics of an animal's immune system structure. Global ASF situation continues to deteriorate and threaten the world's food security. Currently, over 50 countries are affected by the disease, and more countries are constantly getting added to the list. In 2019, African swine fever was reported in Mongolia, Vietnam, Cambodia, North Korea, South Korea, Laos, Slovakia, Serbia, Myanmar, Indonesia, the Philippines and Timor. Greece, Papua New Guinea, India and Germany joined the ranks of ASF infected countries in 2020, Malaysia and Bhutan – in 2021. Thus, the disease continues to spread and affect the countries located on various continents and differing in population size, agriculture and veterinary legislation development levels. ASF-associated losses increase with the growing number of the countries affected with the disease. In particular, the ongoing epizootic in China, with more than 100 million pigs destroyed and dead, has had a serious impact on the global pig production sector [3–5].

Despite the recent progress achieved in the studies of the ASF agent and the development of specific ASF prophylaxis means, there are still no commercially available and effective vaccines. One of the reasons for this is the insufficient knowledge of ASF pathogenesis and immune defence against the disease. At the same time, ASF virus itself possesses a number of mechanisms to escape the carrier's immune response, which include the ability to effectively replicate in macrophages, to alter cytokine and interleukin production, as well as to escape neutralization by specific antibodies [6, 7].

Studying these processes involves the use of ASF virus strains that differ in virulence, contagiousness, reactogenicity, the severity of induced clinical signs and include both naturally attenuated strains and those attenuated by a long-term passaging of the virus in cell cultures. Such strains are necessary for the identification of ASFV virulence and pathogenicity factors, as well as for the development of vaccines based thereon [8–11]. However, according to the literature data, the ASFV population, even within the same geographic region or a large disease outbreak area, may not be homogeneous as regards its biological properties and can simultaneously comprise the strains differing in virulence and hemadsorption activity in cell cultures. This is indirectly evidenced by the data from the studies of the isolates recovered from ticks, as well as by the increasing number of seropositive sample detections in boar in Eastern Europe [12, 13]. Obtaining reliable results requires the analysis of properties of the virus material samples that are homogeneous by composition, rather than of the entire heterogeneous population of the virus. Therefore, researchers are facing the task of

isolating clones or pure viral lineages being homogeneous as regards their biological properties.

All the methods used for the isolation of such clones are based on the separation of a heterogeneous virus mixture into individual samples and the determination of their biological properties for the identification of virus subpopulations followed by their isolation and the propagation of single infected cells or viral particles. However, conventional culture-based methods for viral particle cloning are not without certain disadvantages; in particular, they are rather time- and labour-consuming. Thus, in order to simplify and automatize the selection and cloning of virus infected cells, it is appropriate to apply flow cytometry cell sorting. Cell sorting can also be applied to enrich or purify cell preparations within various research activities, and cytometry can also be utilized for absolute cell count determination in a tested sample using special calibration counting particles or by a volumetric method [14–16].

To achieve all these purposes, different methods based on certain physical principles, which can be classified as active and passive, are applied. Active systems typically use external forces (acoustic, mechanical, electric, magnetic and optical) to displace cells for sorting, whereas passive systems use inertial forces, filters with different pore sizes and adhesion mechanisms [16–18].

Fluorescence-activated cell sorting (FACS) allows for the detection and purification of specific cell populations based on their phenotypic markers, such as relative size, granularity, the presence (on their surface or in the space delimited by a cell membrane) of specific clusters of differentiation identified by flow cytometry. This technique enables researchers to get a better insight into the characteristics of the target population without any impact of other cells.

At present, fluorescence-activated cell sorting methods are automated and robust. Modern flow systems are able to analyze and sort over 50,000 particles per second based on various criteria. In conventional FACS systems, fluorescently labeled cells organized in a laminar flow stream pass through a focused laser beam that scatters into one or more photodetectors. Then the fluorescent signal is analyzed to assign a certain cell type to each registered event of interception with the laser beam. After being typed, each single cell can be deposited into an individual well of the culture plate through discrete sorting. A particle is encapsulated into an aerosol droplet that is charged. When a charged droplet passes through charged plates, it is electrostatically sorted [14, 16].

The sensitivity of FACS is so high that it even allows the sorting of single cells for their subsequent sequencing. This technique is, nevertheless, not without disadvantages. Sorting can be hampered when the cells or particles of interest have a high level of autofluorescence. Besides, the binding of fluorescently conjugated antibodies with their specific ligands located outside the cells can alter the functional activity of sorted cells, thus influencing the results of further experiments. Therefore, the methods based on cell assessment by their size and granularity were selected for the primary sorting of the ASFV infected cell culture. And last but not least, the appropriate operation of a flow sorting system requires, in most cases, the involvement of a highly qualified personnel [17].

It should be noted that fluorescence-activated cell sorting can be applied not only for cells, but also for other discrete particles, including intracellular vesicles, and even for individual virions. However, the translation of this idea into practice raises certain difficulties related to the specific features of virion morphology and the configuration of the optical system of the instrument used [18–22].

First of all, the virus should be propagated to sufficient titres in the cell culture, then the infected cells should be destroyed to release virions. Different methods, such as a freeze-thaw cycle, ultrasound sonication, cell membrane lysis or osmotic pressure modification, can be employed to destroy cells [23]. As a result, a suspension containing residual live, dead and dying cells, cell debris of variable size and virions is formed. The fraction of virions will also have a heterogeneous composition, since it will contain both mature virions capable of infecting live cells and immature virions lacking infectivity, as well as destroyed fragments of virions, released viral nucleic acid and incompletely assembled empty viral capsids. The studies of ASF virus will add to this list the mature virions that have acquired a supercapsid envelope made of the cell membrane after complete budding from the cell. Thus, the virus-containing suspension resulting from cell destruction requires further purification from residual cell debris and nonfunctional virions.

In addition to the above, the sorting of individual virions is limited by the specific characteristics of the optical system configuration of the sorter used. In view of the fact that the virion is as small as a few hundreds of nanometres, whereas average eukaryotic cell sizes are from 10 to 50 μm, the detection of an individual virion requires the use of a highly sensitive detection system that is not only able to register even the smallest changes in laser beam brightness caused by a viral particle passage through it, but also has a high signal-to-noise ratio necessary for effective identification of the signals of interest from the background noise, the presence of which is unavoidable during the analysis due to a number of physical factors, in particular the mutual overlapping of fluorescence emission frequencies of the dyes applied, the electronic noise of sensors and the scattering of light encountering water molecules. The use of fluorochrome labelled antibodies to the virus cannot fully solve the problem of individual virion detection, since, due to the small area of the virion surface, only a limited number of dye-conjugated antibodies can physically fit onto it. The size of a mature ASFV virion does not exceed 200 nm. Besides, the said antibodies will bind with their specific ligands located not only on the mature and infectious virions, but also on the surface of empty virions and especially of fragments thereof, which could result in an increased background fluorescence level and hindered detection [22, 24].

In the light of the above, this study was aimed to perfect the procedure for the sorting of single ASFV infected cells from porcine bone marrow (PBM) and continuous African green monkey kidney (CV-1) cell cultures being heterogeneous by composition into the 96-well plates to select the most promising clones of the virus.

MATERIALS AND METHODS

The infection of susceptible cell cultures involved the use of African swine fever virus "ASF/ARRIAH/CV-1" strain prepared by the adaptation of ASF virus "8 No. 2/Odintsovo-02/14" strain subjected to serial

passages in the continuous CV-1 cell culture. This strain shows a moderate infectivity and can accumulate in PBM and CV-1 cell cultures at titres of 6.0 to 7.0 lg $HAdU_{50}/cm^3$; however, its lethality for pigs of any age group does not exceed 37.5%. It is also important to note that this variant of the virus has retained the ability to infect primary porcine cell cultures [25].

The cultivation of "ASF/ARRIAH/CV-1" strain in the primary and continuous cell cultures was performed at the FGBI "ARRIAH" under the laboratory conditions appropriate for the handling of pathogenicity group II–IV agents. Freeze-dried ASF virus ASF/ARRIAH/CV-1 strain was obtained from the State Collection of Microorganism Strains of the FGBI "ARRIAH". The following two cell cultures were used for the works performed: a primary PBM cell culture grown in Eagle's nutrient medium supplemented with 20% (v/v) fetal bovine serum and a continuous CV-1 cell culture grown in Eagle's nutrient medium supplemented with 10% fetal bovine serum.

To prepare an infectious virus-containing fluid, ASF virus "ASF/ARRIAH/CV-1" strain was cultivated in the 25 cm^3 plastic culture flasks at +37 °C. After a 72-hour incubation of the ASFV infected cell culture, the monolayer was harvested using trypsin solution, transferred to centrifuge tubes and centrifuged to pellet the cell debris with subsequent removal of the supernatant and resuspension in the normal saline solution. The prepared samples were used for sorting.

The virus titre in the tested sample was determined with hemadsorption (HAD) test according to the standard procedure. The infectious titre of the virus was calculated according to the Karber or Reed and Muench method and expressed as lg $HAdU_{50}/cm^3$ [25].

To perform polymerase chain reaction (PCR) testing, the "Test System for African Swine Fever Diagnosis with Real-Time PCR Coupled with Fluorescence Detection" (FGBI "ARRIAH") was applied according to the manufacturer's instruction.

The cells were sorted using a properly calibrated MoFlo Astrios EQ sorter (Beckman Coulter, USA).

The discrimination of the so-called cell doublets being the aggregation of two or more cells, especially when testing poorly disaggregated samples, as well as when using higher liquid flow rates for sorting, was performed through the analysis of pulse height, area and width with the appropriate software.

RESULTS AND DISCUSSION

Primary porcine cell cultures are commonly used for ASFV-related studies, since the ASF agent can be reproduced in them without any preliminary adaptation. For this study, a primary PBM cell culture was chosen; it has a high susceptibility to ASF virus, and the virus replication in this cell culture is accompanied by the occurrence of hemadsorption and the destruction of infected cells.

At first, at least 50 thousand events (photodetector responses to changes in laser beam intensity as a result of particle passage) were collected, then cell doublet discrimination was performed, the cell subpopulation was gated (R1) based on forward versus side scatter parameters, and the cells were sorted onto a slide for microscopic examination at 460× magnification. At this stage,

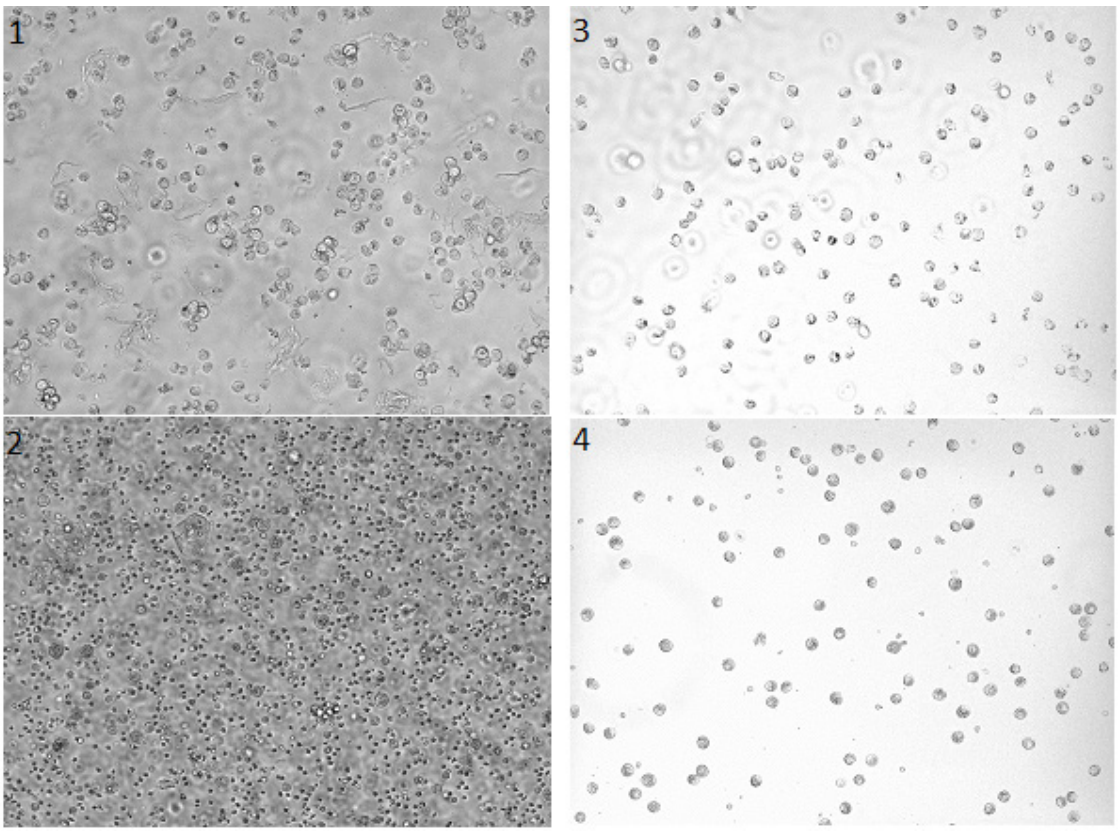

Fig. 1. Cell suspension micrographs taken through an optical microscope (460× magnification) before (1; 2) and after (3; 4) its sorting: upper images – PBM cell culture; lower images – CV-1 cell culture

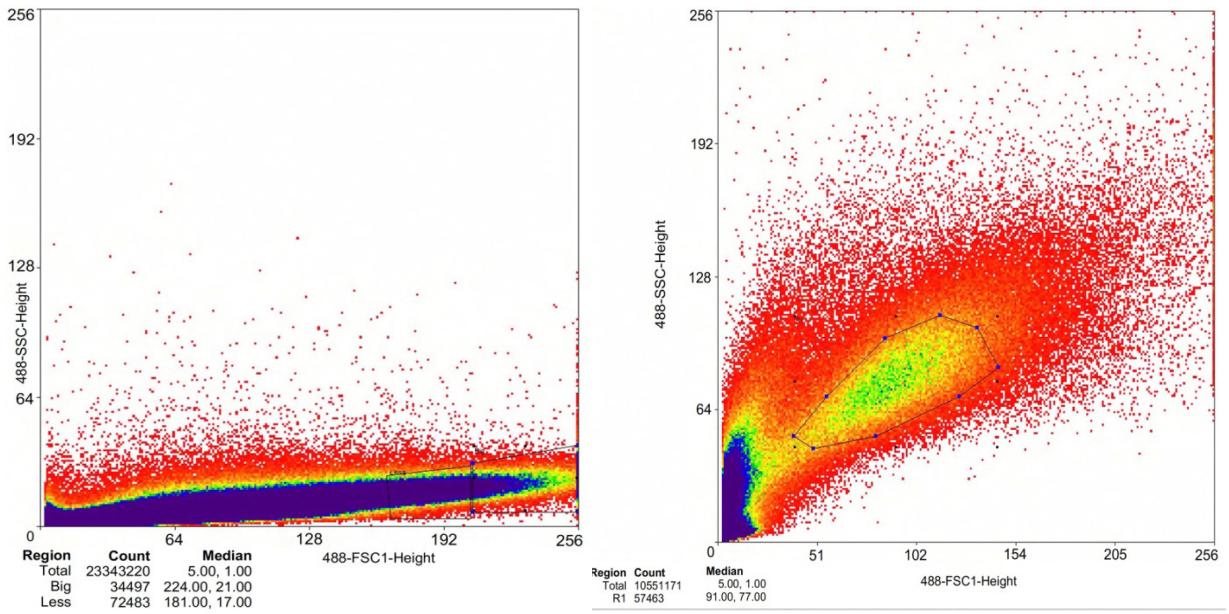

Fig. 2. Side (ordinate) versus forward scatter (abscissa) plots for PBM cells (left image) and CV-1 cells (right image). Gated regions are shown

to accelerate sorting, the sorter was set for lower purity that enables the sorting of droplets containing double or misplaced particles. As the findings presented in Figure 1 show, the resulting suspension has a practically homogeneous cell composition as regards morphological characteristics.

This was followed by single cell sorting into a 96-well culture plate containing Eagle's medium supplemented with 20% bovine fetal serum (0.01 cm^3 per well), 16 wells served as controls. The sorter was set for higher purity so that it could abort droplets containing several particles or an off-centre particle, which corresponds to Single 0.5 sorting mode. The gated region is shown in Figure 2.

In order to isolate and clone new variants of the attenuated ASF virus with stable cultural and biological properties for the further studies of specific features of ASF-associated immunogenesis and the implementation of genetic modifications, sorting of the virus adapted to the CV-1 cell culture was performed using the same sorting parameters.

After PBM cell culture sorting, the HAD test detection rate of ASF positive wells was 30%. Since hemadsorption only occurs in primary cell cultures, CV-1 cells were tested for virus genome fragments with PCR. Based on the PCR test results for the samples prepared using CV-1 cell culture, positive wells were detected in 20% of cases. The detection of negative wells can be attributed to possible sorting of uninfected cells or cells with the virus that has lost its infectivity into these wells. The fact that cell sorting involves the application of mechanical forces to the cells, which increases a cell damage risk, should also be taken into consideration [16].

Besides, aerosol generation at the time of sorting can potentially lead to ASF virus introduction into inappropriate wells. However, the virus was not found in the intact controls of the plate (16 wells) during our experiment.

The virus clones obtained as a result of sorting were propagated in the continuous and primary cell cultures, and this allowed to confirm the presence of the inactivated ASF virus in the selected cells. The virus propagated from single sorted cells to the sufficient amounts was used for a bioassay in naturally susceptible animals.

CONCLUSION

Thus, flow cytometry sorting of cells based on their physical parameters (size and granularity) allows for the preparation of homogeneous enriched cell suspensions. The method makes it possible to clone the virus rapidly and effectively through the sorting of infected cells into a 96-well plate. However, maximum effectiveness would be achieved through using fluorochrome labelled ASFV antibodies to directly sort its extracellular virions, and this is the aim of further work of the specialists of the FGBI "ARRIAH" Reference Laboratory for African Swine Fever.

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Morphological features of bovine placenta in case of viral, bacterial and protozoal infections

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SUMMARY
The problem of the intrauterine infection of fetus is one of the most critical ones in veterinary obstetrics and in perinatology due to the high level of infection in pregnant cows, the risk of developmental disorder of fetus and the birth of sick calves. Complications of pregnancy occur in case of viral, bacterial and protozoal infections, when the pathogen enters the uterus in an ascending or descending way with further transplacental infection of the fetus. Morphological studies of placenta of Black Pied cattle infected with bovine viral diarrhea, chlamydia and neosporosis were carried out. The presence of the pathogen was confirmed by serological and molecular genetic methods. The material used for histological studies was the fetal part of placenta. After sampling, the material was fixed in a 10% neutral formalin solution, then xylene-free method for histological preparation was used. Afterwards, samples were embedded in paraffin. In order to study morphological structures, samples were sectioned at 5–6 μm, and stained with hematoxylin and eosin. Histological sections were analyzed using a Leica DM 1000 light microscope at a magnification of 100×, 200×, 400×, 630×. On the basis of the conducted studies, it was established that bovine viral diarrhea-associated morphofunctional changes in the "mother – placenta – fetus" system are characterized by involutive-dystrophic changes with microcirculation disorders and the development of an immunity-associated inflammatory process. *Chlamydia abortus* intrauterine infection in the "mother – placenta – fetus" system in cows causes a complex of destructive morphological and functional changes of an infectious and toxic nature with a pronounced inflammatory reaction, involvement of blood vessels in the pathological process, and endothelial dysfunction development, alongside with tissue necrosis in case of a chronic process. The presence of cellular structures in the placenta and the inner part of the umbilical cord is a pathognomonic sign of chlamydia. The role of transplacental transmission of *Neospora caninum* in cattle was confirmed, the *Neospora* parasites subjected to basophilic staining were detected not only in the tissues of the placenta, but also in histological sections of the fetus heart and liver. The main characteristic diagnostic sign is the presence of basophilic stained *Neospora* parasites in the organs of the mother and fetus, placenta, and intervillous space. As part of the study morphological features of placenta, one of the most unique histohematic barriers and the basic element of the intrauterine infectious process, were determined.

Keywords: intrauterine infection, placenta, fetoplacental complex, bovine viral diarrhea, chlamydiosis, neosporosis, morphological changes

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Морфологические особенности плаценты крупного рогатого скота при вирусных, бактериальных и протозойных инфекциях

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РЕЗЮМЕ

Проблема внутриутробного инфицирования плода является одной из ведущих в акушерской практике и перинатологии в связи с высоким уровнем инфицирования беременных коров, опасностью нарушения развития плода и рождения больного теленка. Осложнения беременности возникают при вирусных и бактериальных инфекциях, а также внутриутробных протозоозах, когда возбудитель проникает в матку восходящим или нисходящим путем с дальнейшим трансплацентарным инфицированием плода. Проведены морфологические исследования плаценты коров черно-пестрой породы, инфицированных возбудителями вирусной диареи крупного рогатого скота, хламидиоза и неоспороза, наличие которых подтверждали серологическими и молекулярно-генетическими методами. Материалом для гистологических исследований служила плодная часть плаценты. Фиксацию материала после отбора осуществляли в 10%-м растворе нейтрального формалина, затем использовали метод проводки этиловый спирт – ксилол с последующим заключением в парафин. Для изучения морфологических структур изготовленные срезы толщиной 5–6 мкм окрашивали гематоксилином и эозином и проводили анализ на световом микроскопе Leica DM 1000 при увеличении 100×, 200×, 400×, 630×. Установлено, что морфофункциональные изменения в системе «мать – плацента – плод» при вирусной диарее крупного рогатого скота характеризуются инволютивно-дистрофическими изменениями с нарушениями микроциркуляции и развитием воспалительного процесса на иммунной основе. При внутриутробном инфицировании *Chlamydia abortus* в системе «мать – плацента – плод» у коров наблюдается комплекс деструктивных морфологических и функциональных изменений инфекционно-токсического характера с выраженной воспалительной реакцией, вовлечением в патологический процесс кровеносных сосудов и развитием эндотелиальной дисфункции с некротизацией тканей при хронизации процесса. Наличие ячеистых структур в плаценте и внутренней части пупочного канатика является патогномоничным признаком хламидиоза. Подтверждена роль трансплацентарной передачи *Neospora caninum* у крупного рогатого скота, базофильно окрашенные неоспоры выявлены не только в тканях плаценты, но и в гистологических срезах сердца и печени плодов. Характерным диагностическим признаком является наличие базофильно окрашенных неоспор в органах матери и плода, плаценте и межворсинчатом пространстве. В ходе исследований определены морфологические особенности плаценты, являющейся одним из самых уникальных гистогематических барьеров и основным звеном реализации внутриутробного инфекционного процесса.

Ключевые слова: внутриутробные инфекции, плацента, фетоплацентарный комплекс, вирусная диарея крупного рогатого скота, хламидиоз, неоспороз, морфологические изменения

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INTRODUCTION

The problem of the intrauterine infection of fetus is one of the most critical ones in veterinary obstetrics and in perinatology due to the high level of infection in pregnant cows, the risk of developmental disorder of fetus and the birth of sick calves [1–3]. Most infectious diseases causing intrauterine infection are subclinical or latent. However, the changes in the homeostasis parameters due to stress or other unfavorable environmental factors induce improper interaction in the "mother – placenta – fetus" system thus causing the disease manifestation [4]. According to modern concept, a combination of adverse external influences contributes to the disruption of the regulation of metabolic and immunological processes in the "mother – placenta – fetus" system, which leads to impaired adaptation to pregnancy, the formation of pathomorphological changes in the fetoplacental complex, the development of multiple organ pathology and a delay in intrauterine development of the fetus or transplant rejection [5–9].

To date, worldwide, the first and the second place in terms of reproductive losses of cattle and the economic damage caused to livestock is given to the protozoal parasites *Neospora caninum* and the causative agent of bovine viral diarrhea [10–17].

A special role in intrauterine infection of the fetus belongs to latent infections under conditions of pathogen-induced immunosuppression. Thus, for example, the causative agents of different infections can balance in the "pathogen – host" system for quite a long time. However, in case of the weakened immune system, *inter alia*, immediate disease clinical manifestation can be observed due to different stress factors.

Complications of pregnancy are associated with viral or bacterial infections, as well as intrauterine protozoal infection, when the pathogen enters the uterus in an ascending or descending way with further transplacental infection of the fetus. Such diseases include: a group of acute respiratory viral infections (infectious bovine rhinotracheitis, bovine viral diarrhea), chlamydia infection and neosporosis of cattle. As a rule, the infectious process affects the placenta, where a complex of degenerative-inflammatory changes develops. To reveal the pathogenetic mechanisms of intrauterine infection of the fetus, it is necessary

to study the complex of pathomorphological changes in the components "mother", "placenta" and "fetus" with different infectants, which will form a scientifically based approach to the development of a system for monitoring and biological protection of animals from pathogens of abortogenic infections.

In view of the above, the research was aimed at the study of morphological changes in the cow's placenta in case of viral (bovine viral diarrhea), bacterial (chlamydia) and protozoal (neosporosis) diseases.

MATERIALS AND METHODS

The tests were performed in the Department of Reproductive Technologies, Federal State Budgetary Scientific Institution "Ural Federal Agrarian Research Centre, Ural Branch of the Russian Academy of Sciences" and in agricultural organizations of the Ural Region.

The object of the study was the placenta of Black Pied cattle infected with pathogens of bovine viral diarrhea, chlamydia infection and neosporosis. Bovine viral diarrhea virus antigens were determined using the IDEXX Bovine Viral Diarrhoea Virus (BVDV) Antigen Test Kit/Serum Plus (IDEXX Laboratories, Inc, USA), antibodies to *Chlamydia abortus* were detected using the IDEXX Chlamydiosis Total Ab Test kit (IDEXX Laboratories, Inc, USA), determination of antibodies to *N. caninum* using the IDEXX *Neospora* X2 Ab Test kit (IDEXX Laboratories, Inc, USA). The results of enzyme immunoassay were evaluated using a SUNRISE reader (Tecan, Austria).

The pathogen DNA was isolated from the biological material and polymerase chain reaction (PCR) was performed in accordance with the manufacturer's instructions for the use of test systems. We used the Diatom™ DNA Prep 200 DNA extraction kit (Isogen Lab Ltd., Russia), the GenPak® DNA PCR test kit for determining the type of species-specific chlamydia infection in cattle for amplifying the DNA of *Chlamydia pecorum*, *Chlamydia abortus*, *Bovine herpes virus/type 1* (Isogen Lab Ltd., Russia), kits for the detection of bovine diarrhea virus (InterLabService Ltd., Russia). For amplification, an Applied Biosystems 2720 thermal cycler (Singapore) was used. The tests were performed by electrophoresis using an agarose gel and a Mini-Sub Cell GT mini-camera (Bio-Rad Laboratories, Inc., USA) with visualization under ultraviolet radiation in a CHEMIDOC XRS+ camera with interpretation of the results using Gel Doc XR+ Gel Documentation System (Bio-Rad Laboratories, Inc., USA). Tests for the DNA of the causative agent of bovine viral diarrhea were performed by PCR with real-time hybridization-fluorescence detection using a Rotor-Gene 3000 amplifier (Corbett Life Science, Australia).

The material for histological tests was the fetal part of the placenta at month 5–6 of gestation (the state of the stroma, chorion villus, intervillous space, vascular component were assessed), and the umbilical cord. After sampling, the material was fixed in a 10% neutral formalin solution, then the ethyl alcohol – xylene wiring method was used, followed by paraffin embedding. To study the morphological structures, the prepared sections 5–6 μm thick were stained with hematoxylin and eosin. Histological sections were analyzed using a Leica DM 1000 light microscope (Germany) at magnifications of 100×, 200×, 400×, 630×.

RESULTS AND DISCUSSION

The study of the relationship between local and general pathomorphological lesions in case of infectious diseases is one of the main conditions for revealing the pathogenetic mechanisms of the onset and development of infection, the characteristics of macro- and microorganism interaction, the immunological reactivity of the body and the course of compensatory-adaptive processes. The morphological assessment of the functional system "mother – placenta – fetus" is of particular interest when infected with pathogens of abortogenic infections and invasions that can pass through placental barrier. In our studies, such pathogens include bovine viral diarrhea, chlamydia, and neosporosis. In conditions where infection of the maternal organism occurs, the likelihood of infection of other components of the system, including the fetus, increases. At the same time, the organs of the animal reproductive system, as well as the placenta, are most susceptible to disruption of morphogenesis and the development of such a predisposition. The study of morphofunctional changes can help to identify the features of the interaction of infectious agents with the host's immune system and determine its role in the disease pathogenesis. In the future, this should become the basis for the development of methods for diagnosing and preventing the development of intrauterine infection.

Viral diarrhea-associated placental pathology in cattle. Histological examination of sections of the fetal part of the placenta revealed involutive-dystrophic changes with circulatory disorders. In the villous chorion there is a pronounced edema of the villous stroma, which has a diffuse character (Fig. 1). In this case, edema is observed both in the syncytiotrophoblast itself and in the perivascular space. The presence of lymphoid cell infiltration indicates the development of inflammatory process including the immunity-associated one. The villous stroma itself is denuded, the epithelial lining of the chorionic villi is disturbed, and in some areas of the placenta there are foci of fibrinoid deposition in the intervillous space and lime salts in the bloodless areas, indicating petrification of tissues in a state of ischemia (Fig. 2). Circulatory disorders are indicated by such characteristic lesions as avascular chorionic villi along with vascular thrombosis (Fig. 3) as well as lesions associated with the destruction of the vascular wall and disorders of cell membrane permeability. Erythrocyte slugging in small blood vessels is observed, as well as intravascular hemolysis (Fig. 4). In this case, hemosiderin deposition is observed both inside the vessels themselves and in the perivascular space (Fig. 5). The accumulation of ferric ions in areas of pathological accumulation of hemosiderin, which have a high catalytic activity, can have a direct damaging effect on the walls of blood vessels, aggravating the development of the pathological process (Fig. 6). The inclusion of reperfusion in the compensatory process against the background of hypoxic disorders contributes to the activation of lipid peroxidation and the formation of active radicals, which is important in the overall picture of the genesis of developing placental dysfunction.

Chlamydia associated placental pathology. During the morphological examination of the fetal part of the placenta from seropositive to *Chl. abortus* cows, the presence of a mesh structure, that is, the denuded stroma, was revealed.

The villous stroma denudation with desquamation of the syncytial epithelium was observed (Fig. 7). Villous syncytium is involved in direct contact with the stroma of the uterine mucosa, provides trophic, transport, gas exchange functions, and also produces a number of biologically active substances and vitamins. Thus, damage to the syncytium is directly detrimental to fetal development. In the intervillous space of the chorion, widespread formations of fibrinoid clots were detected (Fig. 8). Cellular structures with a basophilic crumbly substance, characteristic of chlamydia infection, were constantly encountered in the villous chorion (Fig. 9). These cellular structures are formed from the host cells after the release of elementary bodies as a result of the pathogen development cycle. The presence of such structures is a pathognomonic symptom associated with chlamydia infection. Kochetov V. V. et al. described similar changes [18]. With a long chronic course of the infectious process, focal and extensive necrotic lesions in placental tissues were detected, characterized by rejection of chorionic villi with single and multiple accumulations of lime salts, indicating petrification of necrotic areas (Fig. 10), focal inflammatory polymorphocellular

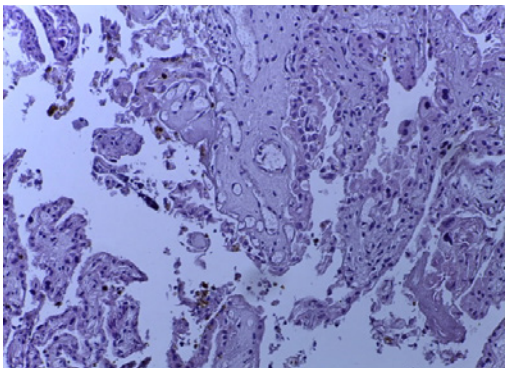

Fig. 1. Placenta. Viral diarrhea-associated edema of villous stroma (hematoxylin – eosin, magnification 200×)

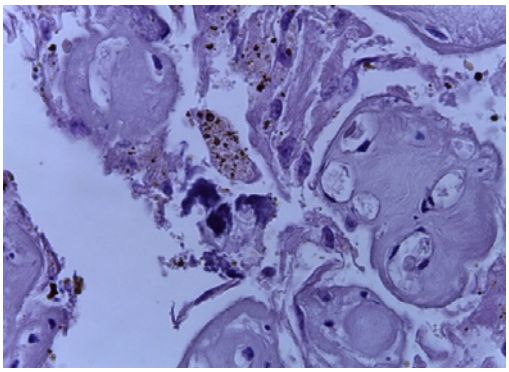

Fig. 2. Placenta. Viral diarrhea-associated lime salt deposition (hematoxylin – eosin, magnification 630×)

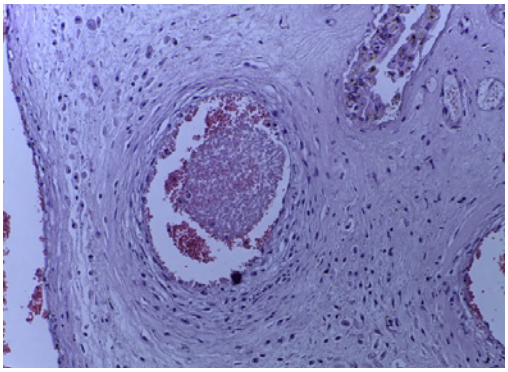

Fig. 3. Placenta. Viral diarrhea-associated thrombus formation in a vessel (hematoxylin – eosin, magnification 200×)

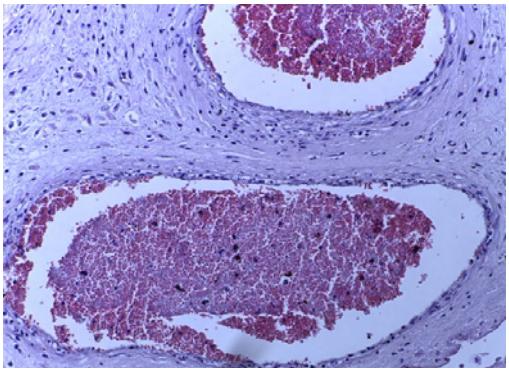

Fig. 4. Placenta. Viral diarrhea-associated hemolysis (hematoxylin – eosin, magnification 400×)

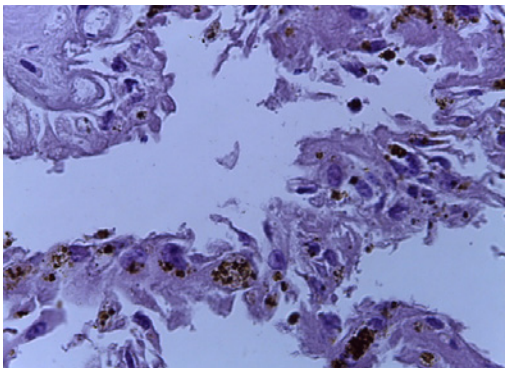

Fig. 5. Placenta. Viral diarrhea-associated deposition of hemosiderin (hematoxylin – eosin, magnification 400×)

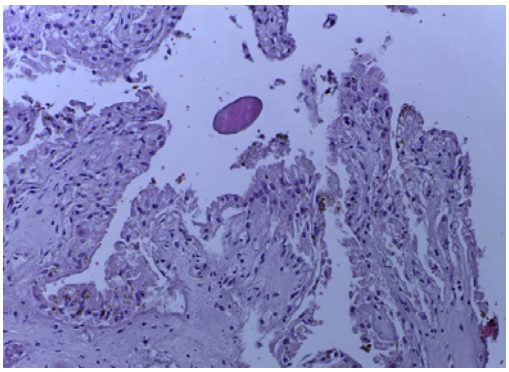

Fig. 6. Placenta. Viral diarrhea-associated fibrinoid deposition (hematoxylin – eosin, magnification 200×)

infiltrates and destruction of the epithelium. In case of improper compensatory-adaptive reactions in response to infection, purulent-necrotic disintegration of the placenta was observed.

To study the pathomorphological features of the development of an inflammatory reaction in case of chlamydia infection, the microvasculature was assessed. When examining the fetal part of the placenta of infected cows, uneven stromal vessel filling was observed. The blood vessels were mostly constricted or contained single erythrocytes. In some parts of the chorion, there was vessel hyperemia with diapedesis of erythrocytes due to the disorders of the vascular wall permeability. In some animals, hemodynamics disorders in the form of thrombosis of the villous capillary network were observed (Fig. 11). In the vascular network of the fetal part of the placenta, the breakdown of erythrocytes was expressed, leukocytes, macrophages, and desquamated endothelial cells were found in vessels of various calibers, which indicates a bacterial infection of the placenta and the development of endothelial dysfunction. There is perivascular polymorphocellular infiltration and

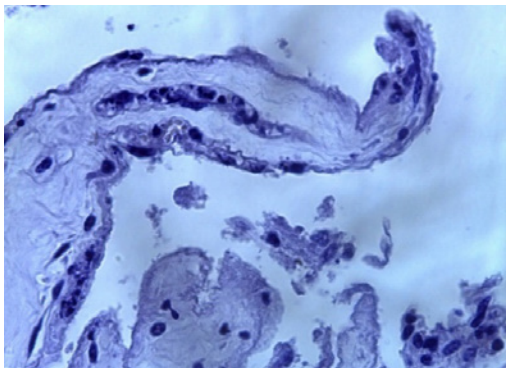

Fig. 7. Chorionic villi. Villus denudation in case of chlamydial infection in cattle (hematoxylin – eosin, magnification 630×)

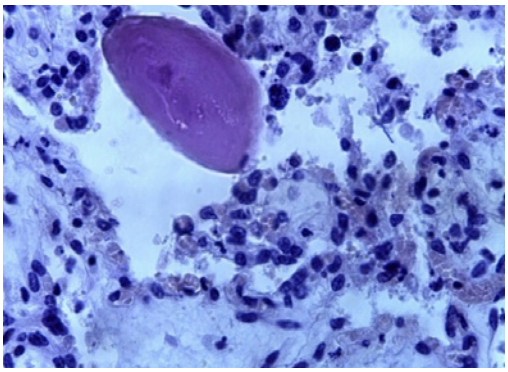

Fig. 8. Chorion. Intervillous space in case of bovine chlamydiosis. Fibrinoid clots (hematoxylin – eosin, magnification 630×)

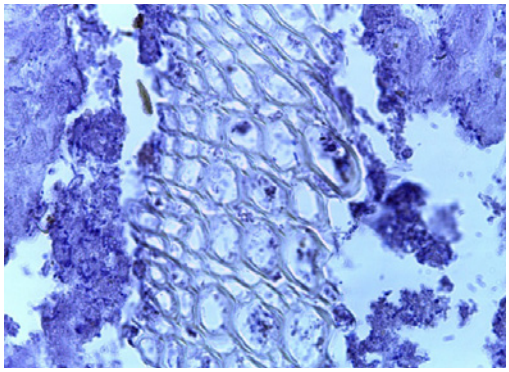

Fig. 9. Villous chorion. Cellular structures characteristic of chlamydia infection (hematoxylin – eosin, magnification 630×)

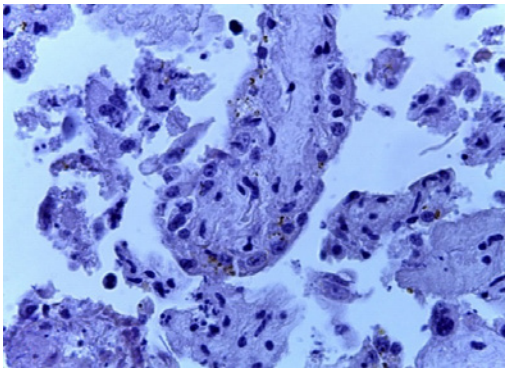

Fig. 10. Chorion. Bovine chlamydiosis-associated focal necrosis characterised by lime salt deposition. Fibrinoid clots (hematoxylin – eosin, magnification 630×)

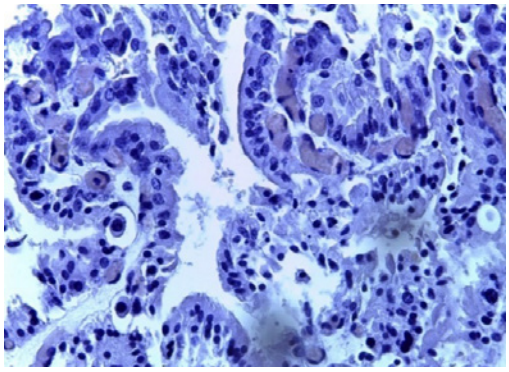

Fig. 11. The fetal part of the placenta. Thrombosis of the capillary network in case of bovine chlamydiosis (hematoxylin – eosin, magnification 400×)

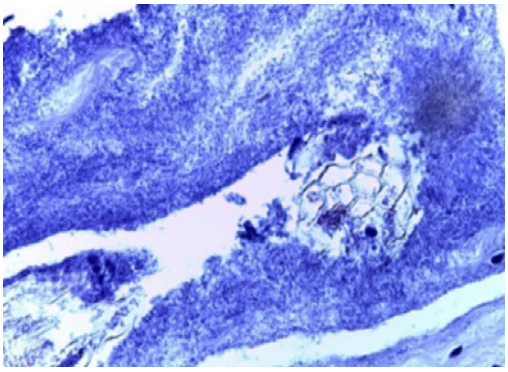

Fig. 12. Umbilical cord. Cellular structures characteristic of chlamydia infection (hematoxylin – eosin, magnification 630×)

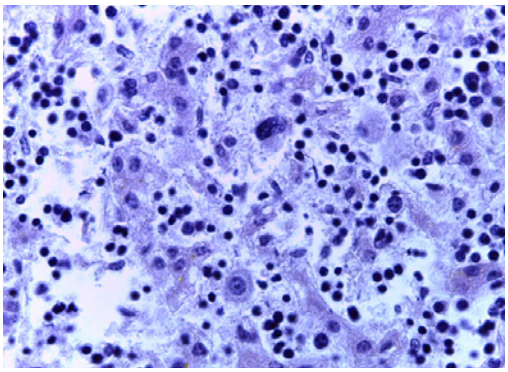

Fig. 13. Placenta. Loose villous stroma. Neospora (hematoxylin – eosin, magnification 400×)

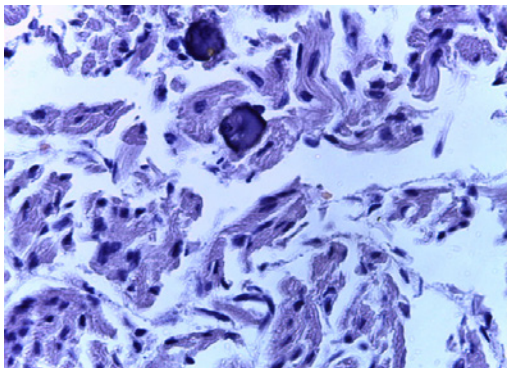

Fig. 14. Placenta. Epithelial layer. Neospora (hematoxylin – eosin, magnification 400×)

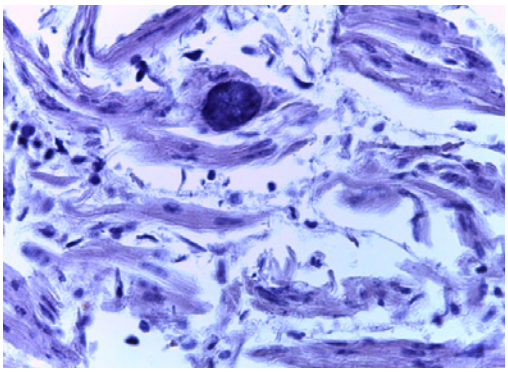

Fig. 15. Placenta. Intervillous space. Neospora (hematoxylin – eosin, magnification 400×)

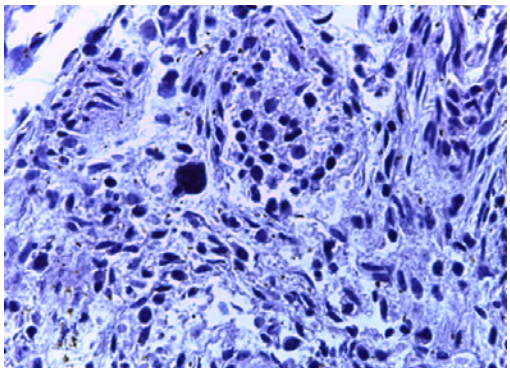

Fig. 16. Fetal part of the placenta. Epithelial layer. Neospora (hematoxylin – eosin, magnification 400×)

the presence of white blood elements in the lumen of the vessels, which is associated with the development of a classic inflammatory reaction in the placental tissues.

Histological examination of the umbilical cord tissue also revealed massive crumbly, basophilic masses with the presence of cellular structures on its inner part, which is typical for chlamydia infection (Fig. 12).

Thus, the conducted studies have convincingly shown that with chlamydia infection in cattle, all three components of the "mother – placenta – fetus" system are affected.

Neospora-associated placental pathology. The development of the placenta in *N. caninum* seropositive cows corresponded to gestation periods of 150–180 days. In the fetal part of the placenta, chorionic villi were preserved, which indicates the absence of a chronic inflammatory process. There were sharply crowded vessels of all calibers: from capillaries to large blood vessels, and some destruction of the surface epithelium connecting the fetal and maternal parts of the placenta, which is typical for spontaneous abortion [19]. Loose connective tissues of the villous stroma were observed in the cows' placenta infected with neospora (Fig. 13). The epithelial layer of the chorion was thickened and loosened. It contained basophilically stained rounded neospora parasites of different thickness (Fig. 14). Some of them were in a state of necrobacteriosis. The same neospora parasites were also found in the intervillous space (Fig. 15). In the epithelial layer of the fetal part of the placenta and the intervillous space, basophilically stained sarcosporidia were also found (Fig. 16), which is typical for neosporosis. In the placenta, signs characteristic of the spontaneous abortion, not associated with the development of a chronic inflammatory process, were observed. Neospora parasites were also found in the internal organs of the fetus, such as the heart and liver. At the same time, morphological signs of internal organ lesions in aborted fetuses were not detected. This is probably due to the fairly developed immune system of the fetuses, whose gestational age in the presented studies was 150–180 days, capable of competently responding to the effects of parasites and limiting their growth.

Thus, the cause of abortions in neospora-infected cows was not associated with the development of the inflammatory process as such, but, most likely, with the immune response of the mother to the invasion of the fetoplacental complex and/or the release of prostaglandins, provoked by the invasion of *N. caninum,* which led to the pregnancy termination. To sum it up the high abortion rate in *N. caninum*-associated pregnancies may be due to several factors. Reactivation of latent infection in seropositive animals is due to the suppression of cell-mediated immunity in mid-pregnancy. Unlike chlamydia, with neosporosis, pathological lesions of the placenta are not so pronounced, and therefore it is difficult to call these lesions the main cause of abortion. Most likely, being localized in the tissues of the placenta, neospora parasites trigger a cascade of reactions associated with the release of prostaglandins, which cause uterine contraction, and pro-inflammatory cytokines, which stimulate the synthesis of matrix metalloproteinases in the trophoblast, which

leads to the destruction of intercellular relationships and, as a result, rejection of the fetoplacental complex. In addition, in case of the general increased sensitization of the body an infested fetus can also induce the process of rejection by the mother's body.

CONCLUSION

On the basis of the studies performed, it was established that morphofunctional changes in the "mother – placenta – fetus" system in case of viral diarrhea of cattle are characterized by involutive-dystrophic changes with microcirculation disorders and the development of an immunity-associated inflammatory process.

In case of intrauterine infection with *Chl. abortus* in the "mother – placenta – fetus" system in cows, there is a complex of destructive morphological and functional changes of an infectious-toxic nature with a pronounced inflammatory reaction, involvement of blood vessels in the pathological process and the development of endothelial dysfunction with tissue necrosis during the chronic process. The presence of cellular structures in the placenta and the inner part of the umbilical cord is a pathognomonic sign of chlamydia infection.

The role of *N. caninum* transplacental transmission in cattle was confirmed; basophilically stained *Neospora* parasites were detected not only in placental tissues, but also in histological sections of the heart and liver of fetuses. Morphological and functional changes in the "mother – placenta – fetus" system in cows in case of *N. caninum* invasion are characterized by signs of microcirculation and hemodynamic disorders, and the development of sensitization. A characteristic diagnostic sign is the presence of basophilically stained *Neospora* parasites in the organs of the mother and fetus, placenta and intervillous space. Neosporosis-associated pregnancy termination, in our opinion, is not associated with the development of the inflammatory process as such, but, most likely, with the mother's immune response to invasion of the fetoplacental complex, which is consistent with the assumptions of a number of foreign authors [4, 17].

The performed studies made it possible to establish the morphological features of the placenta, which is one of the most unique histohematic barriers and the main link of the intrauterine infectious process, which explains the close attention of researchers to this temporary organ.

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The use of Kutikulin for treatment and prevention of gastrointestinal diseases in calves and piglets

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SUMMARY

The global economy dictates more and more stringent requirements for the quality and volumes of products consumed. Veterinary professionals have to look for medicinal products that have a sparing effect on the animal body and, at the same time, are capable of eliminating disease causes. The paper presents the results of tests of Kutikulin, a product made at the Vologda Branch of the FSC VIEV, which consists of chicken gizzard cuticle containing a keratoid secretion and a number of biologically active enzymes. It is a non-toxic, water-insoluble, odourless yellow-green powder with a bitterish taste. When Kutikulin was used to treat newborn calves with a mild dyspepsia resulting from various alimentary causes, the disease duration averaged 2.9 days, recovery rates in the groups were approximately the same (96.6–96.9%). Kutikulin treatment of older calves allowed to reduce the duration of treatment by almost a day, to increase recovery rates by 4.8% and to decrease the number of deaths by 1.6 times. When used for preventive purposes in weaned piglets, Kutikulin helped to decrease morbidity in groups 1, 3 and 5 (test groups) by 2.7, 8.9 and 1.8 times, respectively, as compared with control groups. Its preventive effectiveness was found to be the highest in group 3 (test) piglets that received Kutikulin on a group basis with a liquid feed at a dose of 1.0 g once a day during 3 consecutive days. Along with a shorter disease duration, test group animals also demonstrated less pronounced clinical symptoms. Thus, the use of Kutikulin reduces gastrointestinal disease morbidity and mortality in calves and piglets.

Keywords: treatment, prevention, gastrointestinal diseases, calves, piglets, Kutikulin

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Применение препарата «Кутикулин» для лечения и профилактики желудочно-кишечных заболеваний у телят и поросят

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РЕЗЮМЕ

Мировая экономика диктует все более жесткие требования к качеству и объему потребляемой продукции. Специалисты в области ветеринарии вынуждены осуществлять поиск лекарственных средств, которые оказывают щадящее действие на организм животных, но при этом способны устранять причины заболевания. В данной статье представлены результаты испытания изготовленного на базе Вологодского филиала ФГБНУ ФНЦ ВИЭВ РАН препарата «Кутикулин», состоящего из кутикулы мышечного желудка кур, содержащей кератоидный секрет и ряд биологически активных ферментов, и представляющего собой нетоксичный и нерастворимый в воде порошок желто-зеленого цвета, горьковатого вкуса, без запаха. При применении с лечебной целью препарата

«Кутикулин» новорожденным телятам с легкой формой диспепсии, вызванной различными причинами алиментарного характера, продолжительность болезни составила в среднем 2,9 дня, процент выздоровевших в группах был примерно одинаковый – 96,6–96,9%. У телят старшего возраста лечение препаратом позволило уменьшить длительность лечения почти на сутки, повысить процент выздоровевших на 4,8%, снизить число павших в 1,6 раза. Использование препарата «Кутикулин» с профилактической целью у поросят-отъемышей сократило заболеваемость в первой, третьей и пятой опытных группах по сравнению с контрольными в 2,7; 8,9 и 1,8 раза соответственно. Наиболее высокая профилактическая эффективность установлена у поросят третьей опытной группы, которые получали препарат групповым методом с жидким кормом в дозе 1,0 г один раз в сутки 3 дня подряд. У подопытных животных помимо сокращения периода заболевания отмечали уменьшение степени выраженности клинических симптомов. Таким образом, применение препарата «Кутикулин» снижает заболеваемость и отход телят и поросят при желудочно-кишечных заболеваниях.

Ключевые слова: лечение, профилактика, желудочно-кишечные заболевания, телята, поросята, препарат «Кутикулин»

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INTRODUCTION

Among pathologies reported on animal farms of the Vologda Oblast, maximum economic losses are associated with gastrointestinal diseases that especially affect young animals. The first 10–15 days after birth, in particular the colostral stage, is the most difficult period as regards young animals' survival. The predisposing cause of gastrointestinal disorders (dyspepsias) is decreased body resistance in newborn calves arising from nutritional deficiencies and inappropriate housing conditions of mother cows. Cows receiving deficient diets give birth to calves that demonstrate reduced enzyme secretion by digestive glands and hydrolysis product malabsorption in the intestine. Such animals typically have gastrointestinal disorders. In view of this, the use of products, which stimulate and normalize enzyme secretion by digestive glands, gains a practical importance. The creation of adequate conditions for calving in specially designated pens and subsequent housing of young animals, as well as timely and sufficient colostrum feeding allow to prevent the disease in most cases. At present, tissue-based products are widely used for gastrointestinal disease prevention and treatment in young animals, as well as for the improvement of their performance [1].

Tissue-based products have been known since the first century AD, with the roots of various plants being used as a base for them at that time. Roman scientists Columella, Apsyrtus and Vegetius reported successful subcutaneous administration of hellebore root to cattle and pigs.

The quality and ecology of products for human consumption, freedom from chemicals and balanced diets are of high importance nowadays. Products of animal origin play an essential role in the diet since they are a complete source of protein [2, 3].

Raw material quality is based on multiple factors such as processing, manufacture, safety. But the focus should be on product quality, which initially depends on animals' housing conditions, their diets and treatment prescribed where necessary. It is known that the unlimited use of antibiotics affects product quality. This topic is particularly relevant in the light of the fact that drugs of such kind are commonly used on farms in order to reduce treatment duration, while spending as little money as possible. As a result, certain disease-causing bacteria develop resistance to antibiotics and thus become insusceptible to drugs at the very time when their administration is vitally important. The use of expensive, not readily available antimicrobials has a negative effect on the economic performance of agricultural establishments. It is therefore appropriate to replace them with products that improve digestion and absorption of nutrients in the gastrointestinal tract [4, 5].

Tissue-based products and lysates prepared by hydrolysis from various organs and tissues are widely applied in veterinary practices. In particular, treatment involves the use of tissue-based products prepared from liver and spleen of cattle, testes of male horses and rams. These substances used in minimum quantities to supplement feeds or as mucous membrane applications promote the growth and development of young animals, increase weight gains in fattening livestock. They also have a favourable effect on the body's natural resistance parameters (serum lysozyme and bactericidal activity, phagocytic activity of leukocytes), morphological and biochemical blood profile (erythrocyte and leukocyte counts, hematocrit, erythrocyte sedimentation rate (ESR), hemoglobin and total protein levels) [6, 7].

Tissue therapy is based on the use of preserved tissues and products prepared from them for the treatment of animals, as well as for the improvement of their performance. Tissue-based products contain protein components that stimulate certain body organs and tissues. Their effectiveness largely depends on an animal's species, age, the functional state of its nervous system or specific features of a disease. The use of tissue-based products leads to a 15–30% rise in weight gains in animals and helps to increase meat production [8, 9].

Tissue therapy, in combination with adequate feeding and optimal housing conditions, has a beneficial effect irrespective of the form of the disease – it improves the body's physiological functions, thus helping to cope with pathological processes. Besides, complications and side effects are extremely rare [10].

Biostimulants are widely used for the treatment of obstetric and gynecological disorders in livestock: chronic endometritis, elementary ovarian dystrophy. Tissue-based products used when treating infectious diseases have a systemic stimulating effect on the body, and this is of practical importance for the enhancement of specific immunity factors and a better diagnosis of chronic infectious diseases [11, 12].

Young animals, first and foremost newborn ones, are at high risk of death from dehydration because of inadequate water metabolism regulation arising from incomplete functional development of kidneys. Such animals shall therefore be subjected to treatment without any delay. Sometimes it is enough to administer a normal saline solution, infusions and decoctions of medicinal herbs, compound electrolytic solutions, such as Ringer-Locke's solution, I. G. Sharabrin's solution [13, 14].

Another important aspect is the development of immunity in animals in early life. It is at this time that the enhancement of immune system is indispensable for the improvement of general body resistance to diseases. Considerable attention should be given to the enhancement of body defences, resistance to environmental conditions, while taking into account the length of time from the start of treatment till full recovery. For the improvement of these parameters, the animal body requires nutrients, but feeds consumed on a daily basis or bioadditives do not always contain the sufficient amounts of them. This can be accomplished by using products of plant and animal origin [15, 16].

One of known tissue-based products is the product made according to V. P. Filatov's method. This scientist was the one who began the studies of biogenic stimulants in 1933. Biogenic stimulants are produced as a result of animal tissue preservation at low temperatures and plant tissue preservation in darkness. They are non-protein substances, which are primarily represented by malic, citric, lactic, succinic, carboxylic acids and two amino acids: arginine and glutamic acid.

According to many authors, the range of biogenic stimulant application in veterinary and human medicine is rather wide. Veterinarian K. Kiselev (1898) used male horse testis extract to treat pleuroneumonia-affected horses and succeeded in bringing them to full recovery. Preserved tissues prepared according to V. P. Filatov's method can be used as a separate medicinal product, as well as in combination with antibiotics and vitamins [10, 13, 17].

An experiment was conducted at the Kazan State Academy of Veterinary Medicine named after N. E. Bauman to study the product made according to V. P. Filatov's method. The product consists of tissues of parenchymatous organs (liver, spleen) from healthy livestock, supplemented with trace elements. The experiment was carried out in twelve 3-month-old calves. The product was administered in the middle third of the neck at a dose of 10 ml every 7 days for 4 weeks. Based on the test results, it was concluded that the functional activity of immune system had enhanced, there had been an increase in the blood levels of total protein by 6.7%, phosphorus by 3.9%, calcium by 3.4%, albumins by 5.4%, *α*-globulins by 7.1%, *β*-globulins by 8.3%, as compared with control animals [18].

The Vologda Veterinary Research Station, now known as the Vologda Branch of the FSC VIEV, developed and patented a biostimulant – Splenivit, a bovine spleen extract supplemented with vitamin B_{12}[1]. It is a sterile fluid of a deep brown colour with a reddish hue, which has no toxic and anaphylactogenic properties. Its effect on the general resistance was determined in pregnant sows. The biostimulant was administered to the test group (132 animals) at a dose of 5 ml 30, 20, 10 days before farrowing. The product was not administered to the control group (172 animals).

The number of stillbirths in sows that had received the biostimulant was reduced by half, piglet survival rate at weaning rose by 2.8 times, and weaner output increased by 2.2 animals.

After double administration of Splenivit, 20-day-old piglets demonstrated a significant increase in hemoglobin levels by 14.7%, an increase in erythrocyte counts by 33.3%, total protein levels by 12.3%, *γ*-globulin levels by 67.3%, phagocytic activity and phagocytic index of neutrophils by 13.2 and 16.7%, respectively, whereas blood leukocyte counts and the number of stress-affected piglets decreased by 39.3 and 25.0%, as compared with controls ($P > 0.95–0.99$).

After triple administration of Splenivit, the most significant differences among 30-day-old piglets consisted in an increase in erythrocyte counts by 37.2%, total sugar levels by 13.5%, neutrophil phagocytic activity by 79.6% ($P > 0.95–0.99$), whereas lymphocyte and leukocyte counts and the number of stress-affected piglets decreased by 11.3, 51.0, 50.0% ($P > 0.95–0.99$).

The tests of blood samples from calves, to which Splenivit had been administered three times at a 7-day interval starting from the age of 2 days, showed an increase in the following parameters: hemoglobin levels by 13.6%, serum bactericidal activity by 36.0%, neutrophil phagocytic activity by 77.8%, complement levels by 60.0%, monocyte counts by 53.0%, whereas band neutrophil counts and sialic acid levels decreased by 47.5 and 20.3%, respectively.

The percentage influence of Splenivit within the entire complex of factors leading to changes in hemoglobin levels, serum bactericidal activity, neutrophil phagocytic activity, complement levels, sialic acid levels, band neutrophil and monocyte counts, was 13.6, 13.5, 13.9, 10.0, 14.3, 15.8, 14.9%, respectively ($P > 0.95–0.99$).

Test group morbidity and mortality were 2.2 times lower in calves and, respectively, 2.7 and 4.1 times lower in piglets, as compared with the control groups. Besides, the number of hypotrophic piglets at weaning decreased by 2.5 times. Thus, Splenivit used in diseased newborn calves and piglets enhances body defences, improves growth and development, reduces morbidity and mortality in young animals.

[1] Gorbunov A. P., Masanskaya V. V. Sposob polucheniya biostimulyatora «Splenivita» iz selezenki zhivotnykh = The method for Splenivit biostimulant preparation using animal spleen. Copyright certificate No. 1695869 A1 USSR, Int. A23K1/00 (2000-01-01). Vologda Veterinary Research Station. No. 4753414/15. Date of filing: 30.10.1989. Date of publication: 07.12.1991. Bull. No. 45. (in Russ.)

Another contemporary example of the use of immunomodulators in veterinary medicine is an experiment carried out by the researchers of the Belgorod State Agricultural Academy in treating functional dyspepsia in newborn calves with Thymogen. The calves of one of the test groups were intramuscularly injected with 0.01% Thymogen solution at a dose of 10 ml for 10 days starting from the second day after birth and Pharmasin-50 at a dose of 5 ml for 4 days. As a result, protein metabolism parameters, such as *α*- and *β*-globulin levels, were found to increase by 52.8% by day 10, and this is indicative of Thymogen effect on immune system establishment, general physiological state of animals, normalization of impaired metabolism, as well as of its possible role in disease prevention [19].

The Department of Infectious and Non-Contagious Pathology of the Federal State Budgetary Educational Institution of Higher Education "Ural State Agrarian University" carried out tests of a plant tissue-based product in the form of medicinal herb infusions supplemented with Dorogov's stimulant ASD-2F. The infusion was given to calves once a day during 7 days. Based on the test results, the product demonstrated a high antimicrobial activity against *Escherichia coli* and *Salmonella typhimurium* strains isolated from diseased animals. It was also found that the duration of treatment in calves affected with colibacteriosis and salmonellosis became shorter and their hematological parameters improved [20].

Thus, the development and use of products of animal and plant origin for gastrointestinal disease treatment and prevention are of great current relevance.

Table 1
The results of Kutikulin treatment in calves

Groups	Number of animals	Disease duration, days	Treatment effectiveness		Died	
			Recovered, animals	%	Animals	%
Newborn calves						
group 1 (test)	174	2.9	168	96.6	6	3.4
group 2 (control)	97	2.9	94	96.9	3	3.1
Older calves						
group 3 (test)	13	4.5	12	92.3	1	7.7
group 4 (control)	8	5.7	7	87.5	1	12.5

Table 2
The results of Kutikulin use for prevention purposes in weaned piglets

Groups	Number of animals	Product dose, g	Became diseased		Died	
			Animals	%	Animals	%
group 1 (test)	250	0.5	6	2.4	1	0.4
group 2 (control)	220	–	16	7.3	2	0.9
group 3 (test)	130	1.0	7	5.4	–	–
group 4 (control)	138	–	62	44.9	7	5.1
group 5 (test)	576	0.5	138	24.0	37	6.4
group 6 (control)	580	–	251	43.3	46	7.9

MATERIALS AND METHODS

Kutikulin, a product consisting of chicken gizzard cuticle that contains a keratoid secretion and a number of biologically active enzymes, was developed at the Vologda Branch of the FSC VIEV. It is a non-toxic, water-insoluble, odourless yellow-green powder with a bitterish taste.

Kutikulin was tested for its treatment effectiveness in diarrhea-affected calves of different ages on several farms of the Vologda Oblast. The following groups of animals were formed: two groups of newborn calves under the age of 10 days – group 1 (test, 174 calves) and group 2 (control, 97 calves), as well as two groups of older calves aged up to 30 days – group 3 (test, 13 calves) and group 4 (control, 8 calves). The treatment of animals in all the groups was started at the onset of the first signs of the disease (diarrhea) according to the regimen adopted on the farms. The test group calves were given Kutikulin once a day instead of antibiotics. The product was administered at a dose of 1.5–2.0 g with a liquid provided to the calves in the morning 20–30 minutes before feeding, the treatment period was 3–4 days. Before giving the powder, the amount of milk or colostrum was reduced to half.

Kutikulin was tested for its preventive effectiveness in weaned piglets (1,894 animals) with a view of preventing gastrointestinal disorders during the first days of weaning and adaptation to a new milk free diet. The product was administered on a group basis once a day for 3 days: to one group of piglets – at a dose of 0.5 g with porridge, to another group – at a dose of 1.0 g with a liquid feed, to the third group – at a dose of 0.5 g with a dry feed.

The animals were handled in accordance with European Convention ETS No. 123.

RESULTS AND DISCUSSION

Kutikulin was administered to newborn and older calves affected with mild dyspepsia resulting from various alimentary causes.

The data presented in Table 1 show that the disease duration in newborn calves in both groups averaged 2.9 days, with recovery rates being approximately the same (96.6 and 96.9%). The use of Kutikulin in older calves allowed to reduce the treatment duration by almost a day, to increase the recovery rate by 4.8% and to decrease the number of deaths by 1.6 times.

Kutikulin was tested for its preventive effectiveness in weaned piglets. Test and control group animals were clinically observed from the moment of weaning until the age of 60 days. During that period, the number of diseased piglets demonstrating the signs of diarrhea and that of piglets, which died from gastroenteritis, were recorded.

The test results presented in Table 2 show that, among 250 weaned piglets of group 1 (test) that received Kutikulin with porridge, 6 piglets (2.4%) became diseased and one piglet (0.4%) died, whereas, among 220 weaned piglets that did not receive the product, 16 piglets (7.3%) became diseased and 2 piglets (0.9%) died. Among 130 piglets of group 3 (test) that received Kutikulin with a liquid feed, 7 piglets (5.4%) became diseased, no deaths occurred. Among 138 piglets of group 4 (control), 62 piglets (44.9%) became diseased, 7 piglets (5.1%) died. Among 576 weaned piglets of group 5 (test) that received the product with a dry feed, 138 piglets (24.0%) became diseased and 37 piglets (6.4%) died. In group 6 (control) comprising 580 piglets, 251 piglets (43.3%) became diseased and 46 piglets (7.9%) died.

During the use of Kutikulin, piglet morbidity in groups 1, 3 and 5 (test groups) decreased, as compared with groups 2, 4 and 6 (control ones), by 2.7, 8.9 and 1.8 times, respectively. Its preventive effectiveness was found to be the highest in group 3 (test) piglets that received the product at a dose of 1.0 g with a liquid feed. The piglets that received Kutikulin demonstrated a shorter disease duration and less pronounced clinical symptoms.

At present, there is no unified view on the mechanisms of tissue-based product action. Biogenic stimulants have effect on the body as a whole. This explains a wide span of their impact. Our test results are consistent with the findings of some researchers regarding the improvement of general responsiveness and functional state of the reticuloendothelial system, activation of gastric gland functioning, intensification of immunobiological activity, stimulation of regenerative processes, gas exchange, hematopoiesis and other vital body functions in response to tissue therapy and, in particular, administration of Kutikulin [21–24].

The use of such substances contributes to better growth and development of young stock, increased weight gains, enhanced natural resistance of the body, improved metabolism and reproductive performance in animals. Livestock and poultry morbidity and mortality rates decrease [25, 26].

CONCLUSION

Thus, Kutikulin has a therapeutic and preventive effect against gastrointestinal disorders in calves and piglets, it is easy to prepare and administer and can therefore be recommended for a wide practical application. Kutikulin has a stimulating and normalizing effect in animals with digestive tract malfunction, as well as in piglets during the period of weaning and adaptation to a new milk free diet, helps to decrease animal morbidity and mortality.

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Biochemical blood parameters and level of endogenous intoxication in cows suffering from hepatopathies under heat stress

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SUMMARY

Global warming results in increased extreme weather events, including heatwaves, droughts and floods, which exceed plants' and animals' tolerance thresholds, thus posing a threat to the economy and agriculture. Under these conditions, heat stress becomes a vital problem for animal husbandry. The paper presents the study results of biochemical blood parameters and endogenous intoxication in cows suffering from hepatopathies under heat stress. Based on the calculated temperature-humidity index, it was established that during the summer season in the conditions of the Krasnodar Region lowlands, cows are under heat stress. Using the method of paired comparisons, two groups of animals ($n = 10$) were formed: the first group was a healthy livestock; and the second group consisted of animals suffering from hepatic pathologies. Blood was sampled from all cows at the beginning of the experiment (the first decade of May) and at the end (the last decade of July). Laboratory tests of blood revealed that as the heat stress develops healthy cows show the increase in the protein concentration in blood, and, on the contrary, animals with hepatic pathologies demonstrate the inhibition of protein synthesis. The higher activity of aminotransferases and alkaline phosphatase in the bovine serum in the summer season when compared to the spring season was established. The study of the endogenous intoxication level dynamics in cattle during the development of heat stress, showed that in both groups the concentrations of medium mass molecules (MMM) increased relative to the background data: in the first group (healthy cows) MMM 237 – by 11.8%, MMM 254 – by 14.4%, MMM 280 – by 16.9%; in the second group (cattle with liver pathology) MMM 237 – by 16.9%, MMM 254 – by 20.3%, MMM 280 – by 33%. Thus, under heat stress, the endogenous intoxication in healthy livestock was almost 1.5 times less intense as compared to the animals suffering from hepatopathies.

Keywords: cattle, liver, hepatopathies, biochemical parameters, endogenous intoxication, heat stress

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Состояние биохимического профиля крови и уровня эндогенной интоксикации у коров с гепатопатиями в условиях теплового стресса

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РЕЗЮМЕ

Глобальное потепление приводит к увеличению частоты экстремальных погодных явлений, включая волны жары, засухи и наводнений, превышающие пороги чувствительности растений и животных, что несет в себе угрозу для экономики и сельского хозяйства. В этих условиях тепловой стресс становится актуальной проблемой для животноводства. В статье представлены результаты изучения биохимического профиля и состояния эндогенной интоксикации у коров с гепатопатиями в условиях теплового стресса. На основании расчетных показателей температурно-влажностного индекса зарегистрировано, что в летний период в условиях равнинной территории Краснодарского края коровы находятся в состоянии теплового стресса. По принципу парных аналогов было сформировано две группы животных ($n = 10$): первая – здоровое поголовье, вторая – с патологией печени. Забор крови у всех коров производили в начале эксперимента (первая декада мая) и по его окончании (последняя декада июля). Проведенными лабораторными исследованиями крови выявлено, что при развитии теплового стресса у здоровых коров происходит повышение протеинового спектра крови, а у животных с гепатопатологией, наоборот, наблюдается ингибирование белкового метаболизма. Установлена более высокая активность аминотрансфераз и щелочной фосфатазы в сыворотке крови коров в летний период относительно весеннего. В результате изучения динамики уровня эндогенной интоксикации в организме коров при развитии теплового стресса показано, что в обеих группах концентрации молекул средней массы (МСМ) увеличились относительно фоновых данных: в первой группе (здоровые коровы) МСМ 237 – на 11,8%, МСМ 254 – на 14,4%, МСМ 280 – на 16,9%; во второй группе (коровы с патологией печени) МСМ 237 – на 16,9%, МСМ 254 – на 20,3%, МСМ 280 – на 33%. Таким образом, при тепловом стрессе интенсивность увеличения эндогенной интоксикации у здорового поголовья была почти в 1,5 раза ниже относительно животных с гепатопатиями.

Ключевые слова: коровы, печень, гепатопатии, биохимические показатели, эндогенная интоксикация, тепловой стресс

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INTRODUCTION

Human-induced climate change is causing dangerous and widespread disruption in nature and affects the lives of billions of people around the world. According to the data presented in the report of the Intergovernmental Panel on Climate Change, the last seven years have been the warmest on the planet, and each year has been warmer than the previous one on record. Scientists say that in the near future this trend will continue, and by 2030 the Earth's temperature will rise by 1.5 degrees. In addition to warming, all natural systems become imbalanced, leading to a change in the precipitation regime, to temperature anomalies and an increase in the frequency of extreme events such as hurricanes, floods and droughts [1–4].

Climate warming poses a threat to the economy and agriculture, as increased heatwaves, droughts and floods are already exceeding plants' and animals' tolerance thresholds. Under these conditions, heat stress becomes a vital problem for high-yielding dairy production.

Heat stress is the result of an imbalance between metabolic heat production inside the animal body and its dissipation to the surroundings. High air temperature combined with increased or, conversely, very low humidity can lead to the heat stress development. The thermoneutral zone of modern cattle breeds ranges from 4 to 20 °C, and of high-yielding breeds – from 9 to 16 °C. The dairy breeds frequently kept in the Russian commercial farms, such as the Holstein, are most adapted to cold weather conditions, but are sensitive to heat. Heat stress in lactating cows reduces the animals' performance due to a decline in milk yields and milk quality, it affects the health status of the animal and shortens the herd life expectancy, which ultimately leads to serious economic losses in livestock production industry [5–11].

In the conditions of the intensive dairy farming, the desire to maximize the performance of cows without appropriate consideration of their physiological needs leads to metabolic reorientation, functional overload of organs and body systems, and primarily of the liver. According to veterinary reports, in recent years, digestive diseases have been on the top positions among the animal mortality reasons and reach 40% in some farms [12–14]. Since the liver plays a significant role in the chemical thermoregulation of the body, the heat stress, especially in animals with hepatopathies, can cause serious overall metabolic disorders often leading to death. Milk productivity in these cows is usually not restored to the initial level, such animals are not fertilized, and they are culled [15, 16].

The state of animals under the influence of stress factors is conditioned by the severity of disorders in various organs and body systems, as well as by the degree of hypoxia and endotoxicosis. Endogenous intoxication syndrome is one of the most common in clinical practice and is observed during a variety of pathologies.

Endogenous intoxication is a process caused by the accumulation of endotoxins in abnormally high

concentrations in tissues and biological fluids (endotoxins are the products of natural metabolism (inflammatory mediators, exo- and endotoxins, products of cellular and protein degradation, etc.), which exceed the natural capacity of the neutralization systems and ultimately damage organs and body systems. In this context, the problem of endogenous intoxication syndrome remains one of the most urgent in medicine, which is associated with the important role of endotoxicosis as a link in pathogenesis and a factor determining the severity of the course and outcome of various diseases. One of the main factors of the endotoxicosis progress is the inability of detoxification systems and organs to cope with toxins, due to adaptive mechanism disruption and morphofunctional disorders of the detoxification organs, and primarily of the liver [17–23].

In this regard, research needed to establish the changes in biochemical parameters and endogenous intoxication level in cows with hepatopathies under heat stress is interesting, and the results will allow to develop effective strategies for pharmacological correction for animals exposed to high environmental temperatures.

The aim of the work is to establish changes in the blood biochemical parameters and the level of endogenous intoxication in cows with hepatopathies under heat stress.

MATERIALS AND METHODS

The studies were performed in a Holstein cattle-rearing farm located in the Korenovsky District of the Krasnodar Region.

The period to study biochemical parameters and the level of endogenous intoxication in the body of cows under thermal stress was determined by a retrospective analysis based on the calculated indicators of the temperature-humidity index (THI) in 2018–2020, taking into account the average daily temperature and environment humidity. The index was calculated using the formula: $THI = 0.8 \times T + (H/100 \times (T - 14.4)) + 46.4$, where T is the environmental temperature (°C); H – relative humidity (%). The interpretation of the results was based on the parameters when the THI less than 68 indicates that the cattle are in the comfort zone; from 72 to 79 means the animals experience moderate heat stress; from 80 to 89 means a severe heat stress; more than 90 means an extremely severe heat stress; over 100 – death is possible.

To conduct studies, two groups of cows (10 animals in each group) were formed in May on the principle of the paired comparisons method. The first group involved healthy cattle, the second group consisted of animals with liver pathologies. Animals were selected for the experiment, rated according to their physiological state (2–3 months of lactation), the results of clinical examination, biochemical blood profile, as well as results of the liver ultrasonography.

Animals were handled in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes ETS No. 123 (Strasbourg, March 18, 1986).

To measure the severity of heat stress in cows, the THI for 5 months was calculated (from April to August 2021).

Blood was sampled from all cows at the beginning (the first decade of May) and at the end (the last decade of July) of the experiment.

The clinical examination was carried out according to the generally accepted scheme, paying special attention to the color of the mucous membranes, the coat condition, the number of ruminal contractions, and liver palpation and percussion to establish the area of hepatic dullness, surface and sensitivity.

Ultrasonography was performed using a PS-380V 5.0 mHz veterinary ultrasound scanner (Russia). The echogenicity, structure and sound conductivity of the liver parenchyma were evaluated.

Laboratory blood tests were performed using an automated biochemical analyzer Vitalab Selectra Junior (Vital Scientific B.V., the Netherlands) and reagents from ELITech Clinical Systems (France) and Analyticon biotechnologies AG (Germany). The thymol turbidity test was performed using reagents from CJSC ECOlab (Russia).

To determine the level of endogenous intoxication, integral biological tests are used, among which an important place is given to the determination of medium mass molecules (MMM) in biological fluids, characterized by one common property – a molecular weight of 300 to 5,000 Da. MMM content in serum was determined using the screening method of N. I. Gabrielyan and V. I. Lipatova [24] at three wavelengths: λ = 237 nm (MMM 237), λ = 254 nm (MMM 254) and λ = 280 nm (MMM 280). To measure the optical density in the ultraviolet region of the spectrum, an Ecoview UV-1100 spectrophotometer (Sanghai Mapada Instruments Co., Ltd, China) was used.

The obtained digital data were processed by variational methods, where significance using Student's *t*-test and the significance the differences between the groups were determined.

RESULTS AND DISCUSSION

The conducted studies established that in the summer season in the conditions of the Krasnodar Region lowlands, cows are in heat stress (THI more than 72), with a majority of cattle suffering from a moderate heat stress (Table 1).

The calculation of the temperature-humidity index for the Korenovsky District of the Krasnodar Region for 5 months of 2021 showed that already in May cows start experiencing heat stress. Subsequently, in almost all periods under study, the animals are in a moderate heat stress (Table 2).

Cows, which demonstrated the following conditions during clinical examination were selected into the group of animals with liver pathology: dull and brittle coat, peeling skin (100% of animals), extensive alopecia (60%); pale mucous membranes (80%), jaundice (20%); forestomach dystonia with rhythmicity and rumination disorder (100%); expansion of the hepatic dullness area and in parallel increased pain sensitivity in 70% of animals, and only one of the symptoms was recorded in the remaining cows (30%).

Ultrasonography of the liver and biliary system confirmed the hepatosis, when the liver is enlarged, the edges of the lobes are rounded, uneven, blurred; the echotexture is coarsened and granular; increased echogenicity and ballooning degeneration of hepatocytes are observed.

During the study period as the heat stress progressed, one cow from Group 2 (the first decade of July) was slaughtered, and post-mortem examination confirmed the diagnosis – fatty liver.

Table 1
Dynamics of the temperature-humidity index from May to August in 2018–2020 in the Korenovsky District, Krasnodar Region

Date	Temperature-humidity index			Average value for 3 years
	2018	2019	2020	
May 1	69.15	65.79	62.82	65.92
May 10	67.32	69.63	64.67	67.21
May 20	70.21	70.73	61.97	67.64
June 1	70.25	**76.57**	70.47	**72.43**
June 10	**74.57**	**76.75**	**74.28**	**75.20**
June 20	**79.09**	**74.92**	**74.14**	**76.05**
July 1	**78.20**	**74.90**	**77.56**	**76.89**
July 10	**77.20**	70.53	**74.88**	**74.21**
July 20	**78.86**	71.70	**74.01**	**74.86**
August 1	**75.55**	71.16	**73.24**	**73.32**
August 10	**74.11**	**75.48**	**72.94**	**74.18**
August 20	**76.43**	**74.51**	**73.42**	**74.79**

Table 2
Dynamics of the temperature-humidity index within the five months of 2021 in the Korenovsky District, Krasnodar Region

Average value	April	May	June	July	August
First decade	53.67	63.72	64.88	**74.17**	**77.06**
Second decade	54.12	67.28	**72.80**	**75.36**	**72.54**
Third decade	58.33	**69.75**	**74.78**	**76.08**	**76.11**
Per month	55.37	67.01	**70.82**	**75.23**	**75.26**

During the study, biochemical tests were performed and cows demonstrating abnormal biochemical blood profile results were selected into the group of animals with liver pathology (Table 3). Thus, the selected animals had a low level of total protein (76.50 ± 2.48 g/L) and urea (2.98 ± 0.07 mmol/L), which indicates a decrease in the hepatic protein synthesis. A positive thymol turbidity test (with a degree of turbidity from + to the maximum value ++++) made it possible to diagnose the inflammatory process in the liver, including in the hepatic parenchyma. The cytolytic syndrome, manifested by the destruction of hepatocyte membranes and the release into the bloodstream of transamination enzymes – transaminases (alanine aminotransferase – ALAT and aspartate aminotransferase – ASAT), was confirmed by the fact that moderate hyperenzymemia was detected in sick cows, the difference in comparison with the healthy cow values was 27.2% for ALAT and 31.3% for ASAT. The activity of alkaline phosphatase (ALP) exceeded the normal parameters and exceeded the enzyme level in healthy cows by 20.2%, which may suggest cholestasis. Hypoglycemia was found in sick animals with a difference of 16.9% in glucose concentration if compared with healthy cows.

The level of endogenous intoxication in cows with liver pathology was higher relative to healthy animals; the difference in MMM 237 was 20%, MMM 254 – 25.1% and MMM 280 – 21%.

The development of heat stress in cows changed the biochemical blood parameters. Thus, in healthy livestock, the amount of total protein in serum increased by 6%. Most likely, the increase in the protein concentration in the blood is the result of the general dehydration of the cows' body under hyperthermia conditions, which leads to blood thickening and increase in total protein in it. It is also possible that in the process of adaptation of animals to high environmental temperatures, an increase in the total protein level in the blood is mediated by corticosterone, which regulates the physico-chemical mechanisms of maintaining blood volume during dehydration due to osmotic pressure. In case of the body overheating, there is probably a disruption in the ureagenesis in the liver, since in the group of healthy cows, despite the increase in total protein, the content of urea in the serum decreased by 26.2% ($p \leq 0.01$) as compared to the baseline values. In animals with hepatopathology under heat stress, further

Table 3
Dynamics of biochemical parameters and endogenous intoxication markers in bovine blood under heat stress ($M \pm m$, $n = 10$)

Parameters	Group 1 healthy animals		Group 2 animals with hepatic pathology	
	start of the study	end of the study	start of the study	end of the study
Total protein, g/L	82.60 ± 3.12	87.90 ± 2.26	76.50 ± 2.48	70.90 ± 0.52*
Urea, mmol/L	5.11 ± 0.15	4.05 ± 0.09**	2.98 ± 0.07	2.64 ± 0.11*
Thymol turbidity test, CU	–	–	+	++
ALAT, Unit/L	29.80 ± 2.13	34.90 ± 0.96*	37.90 ± 2.95	40.80 ± 1.53
ASAT, Unit/L	90.40 ± 5.46	93.70 ± 4.51	118.70 ± 3.54	132.60 ± 5.74
ALP, Unit/L	129.10 ± 3.15	141.80 ± 3.16*	163.20 ± 4.33	184.50 ± 2.50**
Glucose, mmol/L	2.56 ± 0.12	2.47 ± 0.14	2.19 ± 0.19	2.05 ± 0.15
MMM 237, CU	0.638 ± 0.033	0.723 ± 0.021*	0.759 ± 0.024	0.913 ± 0.016**
MMM 254, CU	0.195 ± 0.020	0.229 ± 0.013*	0.244 ± 0.012	0.306 ± 0.019**
MMM 280, CU	0.181 ± 0.012	0.218 ± 0.029	0.219 ± 0.008	0.327 ± 0.014*

Differences are significant: * $p \leq 0.05$, ** $p \leq 0.01$ as compared to the background data.

inhibition of protein synthesis was observed with a significant ($p \leq 0.05$) decrease in total protein by 7.8% and urea by 12.9% in the serum.

Higher aminotransferase activity was established in the serum of cows in the summer season as compared to the spring season. Moreover, the ALAT activity in healthy animals tended to upper reference values and amounted to (34.90 ± 0.96) Unit/L, which is 14.6% higher than the initial data (at $p \leq 0.05$). In cows of Group 2, this value was higher – (40.80 ± 1.53) Unit/L (the difference with the start of the experiment was 7.1%). Increased ALAT activity in the serum is considered as an indicator of the hepatocyte destruction and, possibly, in cows with liver dystrophy, a significant proportion of hepatocytes had already been destroyed, which caused a less pronounced increase in the enzyme in these animals. ASAT concentration in healthy cows practically did not change, and in sick animals increased by 10.5%.

The increase in the ALP activity in all experimental animals was consistent and amounted to 8.9% in Group 1 and 11.5% in Group 2. The increase in the ALP activity can be caused not only by the influence of heat stress, but also by increased activity of both placenta-like (a marker of the feto-placental system normal functioning) and bone-specific (fetal bone matrix maturation and mineralization) alkaline phosphatase. Since all cows selected for the experiment had increased pregnancy periods, hyperenzymemia in this case was of a physiological and adaptive nature due to changes occurring in the body of a pregnant animal.

In all animals the glucose concentration in the serum tended to decrease with the most pronounced changes revealed in Group 2 (difference with the baseline data is 6.8%). The possible pathophysiological mechanism of these changes is conditioned by the inverse relationship between the level of cortisol and glucose, which leads to depletion of glucose and glycogen stores in the liver, as well as a change in the insular apparatus functions when exposed to stress factors.

The study of the dynamics of the endogenous intoxication level in cattle during the development of heat stress, showed that in both groups the concentrations of middle molecules increased relative to the baseline data: in Group 1 (healthy cattle) MMM 237 – by 11.8%, MMM 254 – by 14.4%, MMM 280 – by 16.9%; in Group 2 (cattle with liver pathology) MMM 237 – by 16.9%, MMM 254 – by 20.3%, MMM 280 – by 33,0%. In general, the obtained results confirm the data that long-term exposure to factors which compromise the homeostasis brings the organism to a lower reactivity. Under stress conditions, the organism is challenged by the task to maintain normal homeostasis and optimize it, but with prolonged exposure excessed waste products of normal or impaired metabolism accumulate in the tissues and biological fluids of the body, which cause the development of endogenous intoxication and increased MMM levels in the blood.

CONCLUSION

Thus, the data obtained indicate that in the summer season, in the conditions of the Krasnodar Region lowlands, cattle are constantly in a state of heat stress. With the development of heat stress in healthy cows, the protein concentration in blood increases, which is facilitated by the general dehydration of the animal organism by hyperthermia, leading to blood thickening and an increase in the total protein in it. Cows with hepatopathology under heat stress, on the contrary, demonstrated the inhibition of protein metabolism, which suggest an insufficient protein-syntheses liver function. The higher activity of aminotransferases and alkaline phosphatase in the bovine serum in the summer season when compared to the spring season was established. Changes in the dynamics

of values characterizing the development of endogenous intoxication syndrome under heat stress in dairy cattle were revealed. The intensity of the increase in the concentration of medium mass molecules in the serum of healthy livestock was almost 1.5 times lower as compared to the animals with hepatopathy. Perhaps this is explained by the fact that cows with a diseased liver suffer from insufficient adaptive and compensation body responses and with prolonged stress, there is a significant increase in integral endotoxicosis markers, which serve as an additional cause of various body system malfunctioning. The results obtained can serve as a justification for the development of an effective strategy for pharmacological correction of heat stress in cattle.

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Serological monitoring of avian influenza and Newcastle disease in the Russian Federation in 2020

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SUMMARY
Within the framework of the Rosselkhoznadzor measures aimed at control of highly dangerous diseases and development of timely recommendations for disease prevention and control, 36,986 serum samples to be tested for the presence of avian influenza virus antibodies and 30,325 serum samples to be tested for the presence of Newcastle disease virus antibodies were submitted to the FGBI "ARRIAH" Reference Laboratory for Avian Viral Diseases in 2020. The samples were collected from domestic, wild and synanthropic birds in 60 Subjects of the Russian Federation. As a result of the laboratory diagnosis, antibodies against type A influenza virus were found in vaccinated chickens from two poultry farms in the Primorsky Krai. Typing of sample sera using hemagglutination inhibition test showed that the detected antibodies were specific to the haemagglutinin subtype of the vaccine antigen (A/H9). Antibodies to the H9 subtype avian influenza virus were detected in sera of non-vaccinated geese from two poultry farms in the Kurgan Oblast and from one poultry farm in the Republic of Bashkortostan. As for the backyards where scheduled vaccination against avian influenza A/H5 is carried out, a low level of immunity was seen in the Republics of Adygea and Chechnya (0 and 15%, respectively), while a high immunity level was observed in the Rostov Oblast (74%). High seroprevalence of Newcastle disease virus was found in adult poultry in indoor industrial farms, which was associated with mass vaccination against the disease. In broiler chickens, post-vaccination antibodies were observed, on average, in 44% of the tested sera samples. The antibodies against Newcastle disease virus and avian influenza virus subtype H5 detected in wild and synanthropic birds indicate the circulation of these viruses in the Russian Federation. The insufficient level of post-vaccination antibodies suggests that the risk of epidemic among poultry in industrial poultry farms and backyards remains.

Keywords: monitoring, avian influenza, Newcastle disease, blood sera, antibodies, hemagglutination inhibition test, ELISA

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Серологический мониторинг гриппа птиц и ньюкаслской болезни на территории Российской Федерации в 2020 г.

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РЕЗЮМЕ

В рамках реализации мероприятий Россельхознадзора с целью осуществления контроля за особо опасными болезнями и для своевременной выработки рекомендаций по профилактике и борьбе с ними в референтную лабораторию вирусных болезней птиц ФГБУ «ВНИИЗЖ» в течение 2020 г. было доставлено 36 986 проб сыворотки крови для исследования на наличие антител к вирусу гриппа птиц и 30 325 проб – на наличие антител к вирусу ньюкаслской болезни. Пробы были отобраны от домашних, диких и синантропных птиц из 60 субъектов Российской Федерации. В результате лабораторных исследований антитела к вирусу гриппа типа А были обнаружены у вакцинированных кур из двух птицеводческих предприятий Приморского края. При типировании проб сыворотки крови в реакции торможения гемагглютинации установили, что выявленные антитела соответствовали вакцинному антигену по подтипу гемагглютинина (A/H9). Антитела к вирусу гриппа птиц подтипа H9 были обнаружены в сыворотках крови от невакцинированных гусей с двух птицефабрик Курганской области и одной птицефабрики Республики Башкортостан. В личных подсобных хозяйствах граждан, где проводится плановая вакцинопрофилактика гриппа птиц A/H5, низкий уровень иммунитета установлен в Республике Адыгея и Чеченской Республике (0 и 15% соответственно) и высокий уровень – в Ростовской области (74%). Высокий уровень антител к вирусу ньюкаслской болезни был установлен у взрослой птицы в промышленных хозяйствах закрытого типа, что связано с массовой вакцинацией против данного заболевания. У цыплят-бройлеров отмечали наличие поствакцинальных антител в среднем в 44% исследованных проб сыворотки крови. Выявленные антитела к вирусам ньюкаслской болезни и гриппу птиц подтипа H5 среди диких и синантропных птиц свидетельствуют о циркуляции данных вирусов на территории Российской Федерации. Недостаточный уровень поствакцинальных антител указывает на сохранение риска возникновения эпизоотий среди домашних птиц промышленных птицеводческих предприятий и личных подсобных хозяйств.

Ключевые слова: мониторинг, грипп птиц, ньюкаслская болезнь, сыворотки крови, антитела, реакция торможения гемагглютинации, иммуноферментный анализ

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INTRODUCTION

Avian influenza and Newcastle disease are the most significant, from both epidemic and economic perspective, avian diseases that cause huge damage to poultry farming worldwide [1–5].

Avian influenza (AI) is induced by type A influenza virus belonging to the family *Orthomyxoviridae*. It is a segmented RNA virus that is classified into 18 hemagglutinating (H1–H18) and 11 neuraminidase (N1–N11) subtypes based on the antigenic difference of two surface glycoproteins [6]. Avian influenza virus (AIV) is an important zoonotic pathogen resulting in mass death of affected birds and causing serious damage to industrial poultry farming and small-scale farms [7–10]. Serious diseases were most frequently caused by AIV belonging to subtypes H5 and H7.

In 2020 the World Animal Health Organization received notifications on outbreaks of highly pathogenic avian influenza (HPAI) from 36 countries around the world. The disease was most often registered in Europe. There were 484 notifications of outbreaks in poultry and 618 notifications of outbreaks among wild birds. The vast majority of the disease cases were caused by HPAI virus subtype H5N8. However, outbreaks caused by HPAI virus of subtypes H5N1 and H5N5 among poultry and HPAI virus of subtypes H5N3 and H5N5 among wild birds were also reported. No distinct prevalence of HPAI subtype H5N8 was observed in Asia. There were 228 outbreaks among poultry caused by various variations of HPAI virus subtype H5 (N1, N2, N5, N6, N8), and 68 outbreaks among wild birds caused by HPAI virus subtypes H5N1, H5N6 and H5N8. HPAI subtype H5N8 outbreaks among poultry were also recorded in South Africa. Notifications on HPAI subtype H7 reported in poultry were received from Australia and the USA [11].

Highly pathogenic AIV H5N8 was first detected in industrial poultry in China in 2010 [12]. In July 2020 an outbreak caused by HPAI virus subtype H5N8 was also reported among wild birds in the Russian Federation. Since August, HPAI virus subtype H5N8 infection started to be registered in Russia among domestic birds both in poultry holdings and in small-scale poultry farms. The most frequent outbreak notifications were received from the Subjects of the Russian Federation bordering on Kazakhstan (the Omsk, Tyumen, Kurgan and Chelyabinsk Oblasts) [13]. Besides, single outbreaks were reported in the Volga, Southern, North Caucasian and Central Federal Districts.

Newcastle disease (ND) is an avian viral disease characterized by pneumonia, encephalitis and multifocal hemorrhages of internal organs. The causative agent is an RNA-virus belonging to the family *Paramyxoviridae*. The ND virus is potentially contagious for most avian species, and it often causes mortality of susceptible domestic birds (mainly gallinaceous species) [14]. At least four panzootics

Table 1
Detection of antibodies to NDV in chicken sera submitted from industrial poultry farms, using ELISA and HI test

Federal District	Parent flock, up to 100 days old		Parent flock, over 100 days old		Broiler chickens	
	number of positives / total number of samples	number of p/f	number of positives / total number of samples	number of p/f	number of positives / total number of samples	number of p/f
Central	392/868	11	4,226/4,365	29	288/935	7
Northwestern	574/924	9	530/531	8	233/1165	3
Southern	241/410	6	1,649/1,696	11	219/630	3
North Caucasian	283/450	3	196/200	1	260/320	4
Volga	509/675	7	3,114/3,314	35	1,272/2,302	18
Ural	39/136	2	382/382	9	110/174	7
Siberian	193/370	7	1,097/1,185	18	243/527	7
Far Eastern	208/250	3	882/1,035	7	197/350	4

p/f – poultry farm.

were recognized [15], negatively affecting not only the economy as a whole, but also human welfare due to food supply reduction [16].

According to the veterinary services of the Subjects of the Russian Federation, ND outbreaks were recorded in the Republic of Ingushetia, in the Vladimir and Kursk Oblasts (11 infected settlements) in 2020 [17].

The necessity of avian influenza and Newcastle disease monitoring studies is determined by the risk of introduction of new virus strains into the country, the risk of pathogen introduction into commercial poultry farms, the emergence of epidemics that lead to great economic losses [18].

The aim of the work was to conduct seromonitoring studies among domestic, wild and synanthropic birds within the framework of the Rosselkhoznadzor measures aimed at control of highly dangerous diseases and timely development of recommendations for avian influenza and Newcastle disease prevention and control.

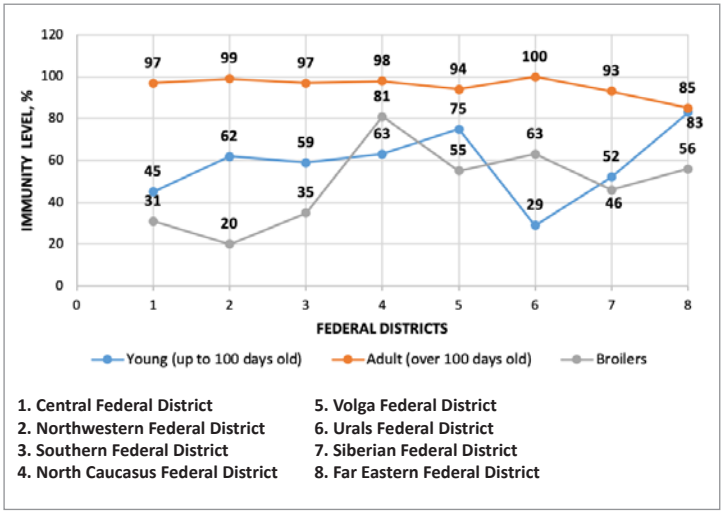

Fig. 1. Intensity of post-vaccination immunity to the ND virus in chickens from industrial poultry farms

MATERIALS AND METHODS

Test samples. Bird sera submitted by the Rosselkhoznadzor Territorial Administrations to the FGBI "ARRIAH" in 2020 were tested.

Test kits and reagents:

– Single serum dilution ELISA kit for detection of antibodies to avian influenza virus (FGBI "ARRIAH");

– Single serum dilution ELISA kit for detection of antibodies to Newcastle disease virus (FGBI "ARRIAH");

– HI test kit for detection of antibodies to avian influenza virus subtype H5 (FGBI "ARRIAH");

– HI test kit for detection of antibodies to avian influenza virus subtype H9 (FGBI "ARRIAH");

– HI test kit for detection of antibodies to Newcastle disease virus (FGBI "ARRIAH");

– reference antigens of AI virus subtypes H5, H7 and H9 and corresponding positive sera produced by IZSVe (Italy);

– reference antigens of AI virus subtypes H5, H7 and H9 and corresponding positive sera produced by GD (Netherlands).

All tests were carried out using commercial ELISA kits according to the manufacturer's instructions. The hemagglutination inhibition assay (HI assay) using reference components was carried out in accordance with the "Methodical Guidelines for HI identification of avian influenza and Newcastle disease viruses"[1].

Treatment of test sera samples. To remove the thermolabile inhibitors, all samples were inactivated in a water bath for 30 minutes at 56 °C.

RESULTS AND DISCUSSION

Pursuant to Rosselkhoznadzor Order No. 1423 of December 25, 2019 "On laboratory testing within the Rosselkhoznadzor activities for ensuring compliance with the World Trade Organization (WTO) Sanitary and

[1] MG 27-16 Methodical Guidelines for HI identification of avian influenza and Newcastle disease viruses: approved by the Rosselkhoznadzor on June 06, 2016. Vladimir: FGBI "ARRIAH". 2016. 14 p.

Phytosanitary (SPS) Agreement requirements upon Russia's accession to the WTO for 2020", 30,325 sera samples collected from poultry, wild and synanthropic birds were tested for the presence of antibodies to the Newcastle disease virus. The samples were received from 60 Subjects of the Russian Federation. The chicken sera were tested using ELISA and HI assay. Serum samples of other species of domestic, wild and synanthropic birds were tested using HI assay.

4,083 serum samples from young chickens under the age of 100 days obtained from 24 Subjects of the Russian Federation were tested. The minimum percentage of seropositive young poultry from commercial and parent stock was in the Urals Federal District, and the maximum percentage was in the Far Eastern Federal District. On average, in the Russian Federation, the immunity level for this group was low and amounted to 60%. Such a low percentage can be explained by the fact that the sera were collected from birds of various ages (1–100 days), and vaccination schemes used in poultry farms do not always provide a sufficient level of antibodies by the time of sampling.

12,708 serum samples obtained from adult commercial poultry in 44 Subjects of the Russian Federation were tested. The maximum percentage of seropositive birds was observed in the Urals Federal District (100%), and the minimum – in the Far Eastern (85%) Federal District. Test results for sera taken from adult commercial poultry in all Federal Districts of the Russian Federation showed persistent and intense immunity (on average 95%).

Tests were conducted for 6,403 serum samples obtained from broiler chickens in 28 Subjects of the Russian Federation. The minimum percentage of seropositive broilers was observed in the Northwestern Federal District, and the maximum – in the North Caucasian Federal District. On average, in the Russian Federation the immunity level for this group was 44%. A low average percentage of positive samples in broiler chicken can be explained by the fact that farms used vaccination schemes that did not allow development of sufficient post-vaccination antibody levels by the time of blood sampling.

The test results are presented in Figure 1 and Table 1.

1,086 serum samples of turkeys, geese and ducks obtained from industrial poultry farms of the Northwestern, North Caucasian, Volga, Urals and Siberian Federal Districts were tested (Fig. 2). The intensity of the immune response exceeding 80% was observed in turkeys in three poultry farms located in the Udmurtia Republic (100%), Orenburg (84%) and Tyumen (83%) Oblasts. The percentage of seropositive birds from the Stavropol Krai, the Republic of Mordovia, Leningrad, Samara and Omsk Oblasts was low and ranged from 0 to 45%.

The low average percentages of seropositive birds among geese (60%) and ducks (32%) can be explained by the fact that not all poultry farms carry out vaccination against the ND virus (according to the accompanying documentation).

In 2020, within monitoring studies to detect antibodies to the ND virus, 5,658 samples of poultry sera were received from backyards and small-scale farms: chickens, geese, ducks, turkeys, quails and guinea fowl. Antibodies to the ND virus were found only in the blood of chickens, geese, quails and turkeys (Table 2).

In 2020, 26,357 samples of chicken sera delivered from 180 industrial poultry farms of the Russian Federation were examined for the presence of antibodies to the AI virus. Four positive samples from Primorsky Krai were detected. As the data in accompanying documents show, the antibodies were induced after immunization with the vaccine against AI subtype H9. No antibodies to avian influenza virus were found in other samples (Fig. 3).

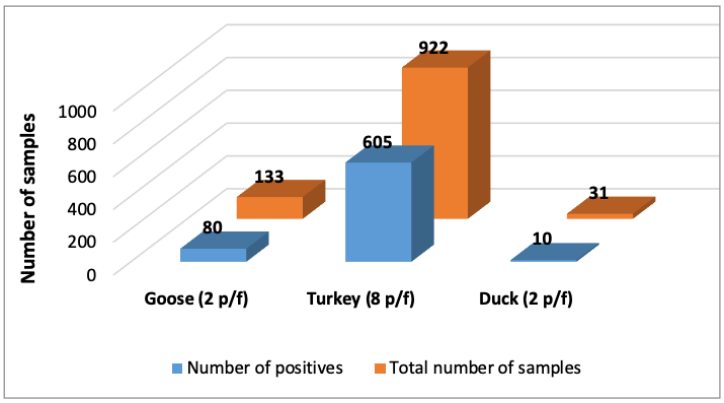

Fig. 2. Detection of antibodies against NDV in turkey, goose and duck sera submitted from industrial poultry farms

Table 2
Detection of antibodies to NDV in poultry sera submitted from backyards and small-scale farms

Federal District	Number of positives / total number of samples			
	chickens	geese	turkeys	quails
Central	191/560	0/10	–	–
Northwestern	28/119	–	–	–
Southern	471/1,588	0/12	3/15	0/10
North Caucasian	674/959	–	50/50	–
Volga	104/203	6/45	12/25	1/5
Urals	24/140	0/39	–	–
Siberian	82/336	3/11	1/3	–
Far Eastern	583/1,116	0/4	225/292	–

"–" no samples were submitted for testing.

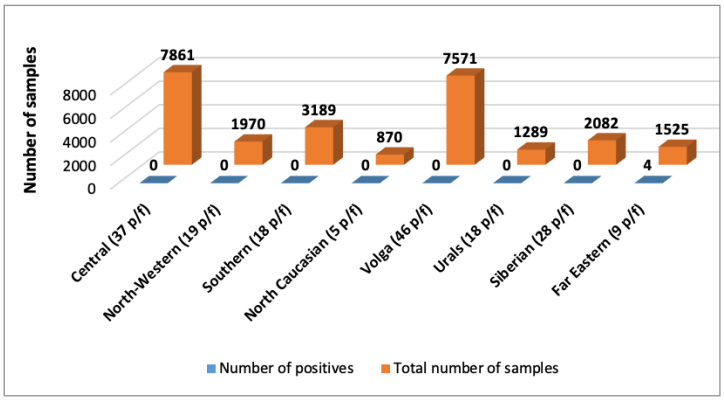

Fig. 3. Detection of antibodies to AIV in chicken sera submitted from industrial poultry farms

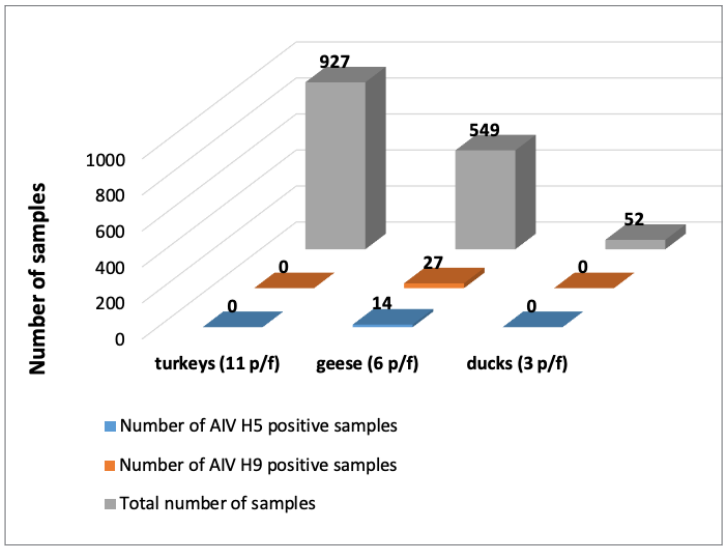

Fig. 4. Detection of antibodies to H5 and H9 AIV in turkey, goose and duck sera submitted from industrial poultry farms

Table 3
Detection of antibodies against H5 AIV in poultry sera submitted from backyards and small-scale farms

Federal District	Number of positives/total number of samples	
	chickens	quails
Central	0/2,334	–
Northwestern	0/119	–
Southern	537/2,122	3/20
North Caucasian	63/959	–
Volga	0/245	0/5
Urals	0/140	–
Siberian	0/336	–
Far Eastern	0/1,642	–

"–" no sera were submitted for testing.

The FGBI "ARRIAH" received 1,528 serum samples from ducks, geese and turkeys from the Northwestern, North Caucasian, Volga, Urals and Siberian Federal Districts. All samples were HI tested for the presence of antibodies to the AI virus subtypes H5, H7 and H9. Antibodies to the AI virus subtype H7 were not detected in all tested samples. No antibodies to the AI virus subtypes H5 and H9 were detected in the sera of turkeys and ducks.

Antibodies to the AI subtype H5 virus were found in the sera of geese received from a poultry farm located in the Kurgan Oblast. According to the data available in the accompanying documentation, the poultry were immunized with a vaccine against AI subtype H5. Antibodies to the AI virus of the H9 subtype were found in sera of geese delivered from two poultry farms of the Kurgan Oblast and one poultry farm in the Republic of Bashkortostan. Vaccination against AI subtype H9 virus on these poultry farms was not stated in the accompanying documentation (Fig. 4).

In 2020 8,704 bird serum samples from backyards and small-scale farms were delivered for monitoring studies to be tested for antibodies to the AI subtype H5 virus. The sera were obtained from chickens, geese, ducks, turkeys, quails and guinea fowls (Table 3). Antibodies to the AIV were found only in chicken and quail sera. According to

Table 4
Detection of antibodies against AI and ND viruses in sera of wild and synanthropic birds using HI test

Federal District	Bird species	The number of positives/ total number of samples			
		ND	AI (H5)	AI (H7)	AI (H9)
Central	synanthropic birds	0/10	0/20	0/20	0/20
	wild birds	0/25	0/25	0/25	0/25
Northwestern	wild ducks	5/120	5/120	0/120	0/120
	crows	4/4	0/4	0/4	0/4
	seagulls	0/16	0/16	0/16	0/16
	wild geese	0/4	0/4	0/4	0/4
	pigeons	10/10	0/10	0/10	0/10
Volga	pigeons	66/80	0/80	0/80	0/80
Siberian	pigeons	35/85	0/85	0/85	0/85
	decorative and wild birds	0/25	0/25	0/25	0/25
	mallard duck	0/3	0/3	0/3	0/3
	wild ducks	0/5	0/5	0/5	0/5

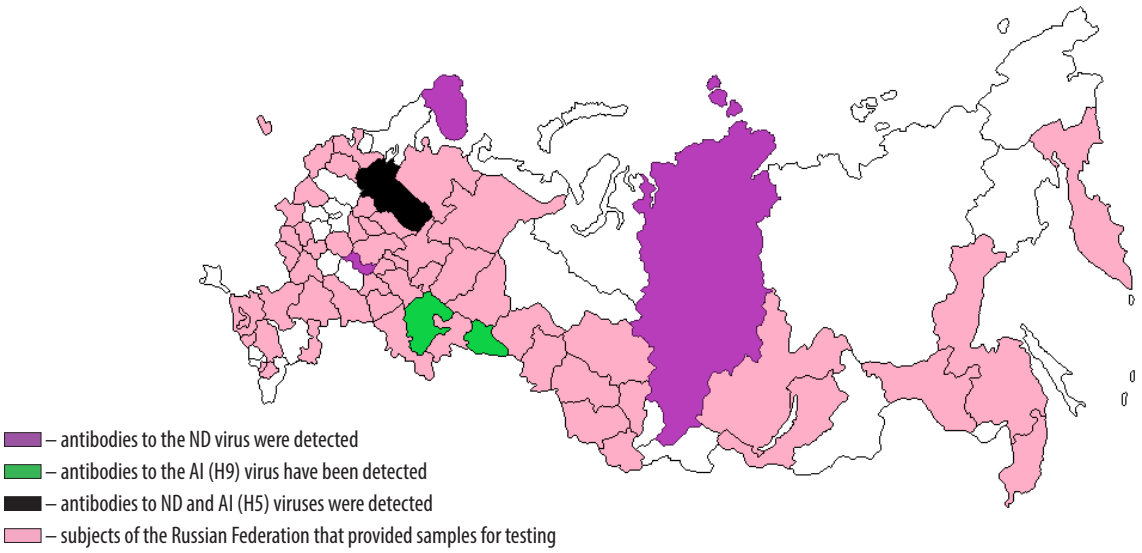

Fig. 5. RF Subjects where antibodies against AI and ND viruses were detected in non-vaccinated commercial, wild and synanthropic birds in 2020

the accompanying documentation, vaccination of chickens against the AI subtype H5 virus was carried out in the Republic of Adygea, the Rostov Oblast and the Chechnya Republic (immunity level in these Subjects of the Russian Federation was 0; 74 and 15%, respectively). Vaccination of quails was carried out only in the Astrakhan Oblast (the percentage of seropositive sera was 30%). No antibodies to the AI subtype H5 virus were detected in the sera from unvaccinated birds.

Many species of wild and synanthropic birds are natural reservoirs of AI and ND pathogens, therefore, monitoring the epidemic situation among birds of this group helps to predict and control the diseases.

In 2020 the FGBI "ARRIAH" received 397 serum samples of wild and synanthropic birds from 9 Subjects of the Russian Federation to be tested for avian influenza. All sera were tested for the presence of antibodies to the AI virus subtypes H5, H7, H9. Based on test results antibodies to the AI subtype H5 virus were detected in 5 wild ducks from the Vologda Oblast. Antibodies to the AIV subtypes H7 and H9 were not detected.

387 serum samples from wild and synanthropic birds were tested for the presence of antibodies to the ND virus. Antibodies to the ND virus were found in 111 sera from pigeons delivered from the Murmansk Oblast, the Krasnoyarsk Krai and the Republic of Mordovia. Antibodies to the ND virus were also detected in sera from wild ducks and crows from the Vologda Oblast (Table 4). The circulation of the ND agent among synanthropic birds, that were, for most part, sampled near poultry farms, indicates a high risk of infection entry in flocks of farmed birds.

Figure 5 shows the location of the Subjects of the Russian Federation, where antibodies to AI and ND viruses were detected in blood sera from unvaccinated commercial, wild and synanthropic birds.

CONCLUSION

Based on monitoring results conducted in 2020, the conclusion can be made on the circulation of avian influenza A/H5 and Newcastle disease viruses among wild and synanthropic birds, as well as avian influenza A/H9 virus among domestic birds in the territory of the Russian Federation. In the future, outbreaks of Newcastle disease and highly pathogenic influenza may occur among birds in the wild population, which also causes a high risk of infection of the above diseases in poultry farms with a low biocontainment safety. Therefore, it is very important to conduct timely monitoring studies to detect the entry of the pathogen and the spread of highly pathogenic influenza and Newcastle disease in wild and synanthropic bird populations, as well as to assess the level of post-vaccination antibodies among domestic birds in order to carry out adequate anti-epidemic and preventive measures.

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Determination of reproductive properties of virulent and vaccine classical swine fever virus strains in primary and continuous cell cultures

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SUMMARY

Classical swine fever (CSF) is a highly dangerous porcine disease. CSF outbreaks are annually notified in several countries. Despite the availability of specific prevention tools, the disease spread risk still persists both at country level and at world level. Hence, the disease surveillance and eradication require highly sensitive methods for early diagnosis of the infection and for tests for the virus circulation in the environment. Development of up-to-date diagnostic methods is based on well-established virus cultivation system; therefore, CSF virus reproduction enhancement, tests of new cell lines without endogenous contamination for their possible use are still of current importance. The said study was aimed at testing of primary and continuous cell cultures for their susceptibility to classical swine fever virus (vaccine virus strains and some field virus isolates recovered in the Russian Federation) and detection of the virus reproduction dynamics with real-time polymerase chain reaction with fluorescent hybridization probes used for detection. Virus replication intensity in primary and continuous cell cultures was also analyzed. The CSF virus was found incapable of replicating in some cell cultures without its preliminary adaptation. Primary porcine and lamb testicle cell cultures grown in minimal essential medium supplemented with 10% normal CSFV-negative porcine serum instead of fetal bovine serum were shown to be useful for the virus accumulation, both for vaccine strains and field isolates. Cultivation parameters and optimal minimal essential medium composition contributing to the 4–10-fold increase in the virus accumulation both in primary and continuous cell cultures were determined.

Keywords: classical swine fever (CSF), CSF virus isolates, vaccine virus strains, cell cultures, real-time reverse transcription-polymerase chain reaction (RT-PCR) with fluorescent hybridization probes used for detection, direct immunofluorescence test

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Определение репродуктивных свойств вируса классической чумы свиней вирулентных и вакцинных штаммов в первичных и перевиваемых культурах клеток

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РЕЗЮМЕ

Классическая чума свиней относится к особо опасным болезням этого вида животных, вспышки которой ежегодно нотифицируют в ряде стран. Несмотря на наличие средств специфической профилактики, опасность распространения заболевания в отдельной стране или во всем мире в целом сохраняется до сих пор. В связи с этим применение высокочувствительных методов ранней диагностики инфекции и определение наличия циркуляции вируса в природе являются необходимыми при надзоре и эрадикации данной болезни. Разработка новейших методов диагностики опирается на хорошо разработанную систему культивирования вируса, поэтому повышение уровня репродукции вируса классической чумы свиней, изучение возможности использования новых, лишенных эндогенной контаминации линий клеток до сих пор остается весьма актуальной задачей. Данная работа посвящена изучению чувствительности первичных и перевиваемых культур клеток к вирусу классической чумы свиней (вакцинных штаммов и ряда полевых изолятов, выделенных на территории России) с детекцией динамики его репродукции при помощи полимеразной цепной реакции с гибридизационно-флуоресцентной детекцией в режиме реального времени. Также проведен анализ интенсивности репродукции вируса в первичных и перевиваемых культурах клеток, при этом установлено, что вирус классической чумы свиней не обладает способностью к размножению в некоторых из них без предварительной адаптации. Показано, что для накопления вируса как полевых изолятов, так и вакцинных штаммов целесообразно применять первичные культуры клеток тестикул свиньи и тестикул ягнят при добавлении в питательную среду 10%-й нормальной серонегативной к вирусу классической чумы свиней сыворотки свиньи, а не фетальной бычьей сыворотки. Определены параметры культивирования и оптимальный состав поддерживающих питательных сред, использование которых способствует увеличению накопления вируса в 4–10 раз как в первичных, так и в перевиваемых культурах клеток.

Ключевые слова: классическая чума свиней, изоляты вируса классической чумы свиней, вакцинные штаммы, культуры клеток, обратно-транскриптазная полимеразная цепная реакция с гибридизационно-флуоресцентной детекцией в режиме реального времени, реакция прямой иммунофлуоресценции

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INTRODUCTION

Classical swine fever (CSF) is a highly contagious infectious disease characterized by fever, blood-vascular system, respiratory and gastrointestinal tract disorders, high morbidity and lethality in domestic pigs and wild boars [1].

According to the World Organization for Animal Health (OIE) data for 2021, only 38 countries located in different continents are CSF free, there are CSF free zones in three countries. CSF situation in Asian and South American countries are complicated.

In the disease-affected countries, CSF causes significant economic losses in pig industry due to decreased animal performance or animal deaths as well as mandatory stamping out carried out for the disease outbreak eradication. Successful pig industry development requires permanent improvement of the available diagnostic test methods, scientifically justified approaches to this highly dangerous disease control and prevention [2–3].

Modern diagnostic methods are essential tools for the agent detection and study of the infectious process progression features in the infected territory. Based on the analysis of currently used methods, they can be classified by either detection target (detection of virus, antigens, antibodies and genome analysis) or test purpose (detection, identification, monitoring, retrospective diagnosis, etc.) [3].

The following main methods are used for CSF virus and CSFV antigen detection: virus isolation in cell cultures followed by detection with direct immunofluorescence test (DIFT), immunohistochemistry (IHC), polymerase chain reaction (PCR) and, of course, bioassay in susceptible animals. Indirect immunofluorescence test (IIFT) is used for retrospective diagnosis and enzyme-linked immunosorbent assay (ELISA) is used for virus-specific antibodies detection [4–5].

In case of CSF suspicion, the disease is finally diagnosed with laboratory tests demonstrating presence of

the agent/genome or specific antibodies when the animals were not vaccinated against CSF [6].

Generally, two or three serial passages in various primary or continuous CSF-susceptible cell cultures are required to increase the pathogen amount in order to overcome sensitivity limit of the diagnostic method used for testing of the sample for the virus [7].

Since the CSF virus has no destructive effect on cells, an additional detection method is used for rapid detection of CSF reproduction in the culture, for example, such as real-time reverse transcription polymerase chain reaction (rtRT-PCR) [8].

Bioassay in susceptible animals is often used for CSF virus isolate diagnosis and tests of CSFV isolates for their biological properties at research laboratories [9].

Virus isolation by inoculation of the potentially virus-containing material in susceptible cell culture is generally used for tests for viable virus. As CSF virus is capable of replicating in continuous porcine kidney cell culture (PK-15), swine peripheral blood leukocyte (PBLs) culture, porcine splenocyte (PS) culture, porcine bone marrow (PBM) cell culture, the CSF virus isolation is carried in one of the said cultures [10].

Since CSF virus does not induce any apparent cytopathic effect (CPE) during its reproduction in different cell lines, additional method is required for detection of the virus reproduction, for example, direct immunofluorescence test (DIFT) or RT-PCR.

Cell cultures are also used for CSF virus accumulation for diagnostic purposes (for antigen scaling-up and analysis of the genome structure, etc.). However, there are some disadvantages when CSF virus is scaled up in primary cell cultures: tissue cell culture preparation is laborious, cell source for the culture preparation is not standardized that hampers reproducibility of individual experiment results, etc.

New effective CSF virus cultivation systems are selected for standardization of diagnostic, virological and molecular-biological tests that allow stable virus antigen and genome accumulation up to the large amounts [11–12].

Owing to live attenuated vaccine development and improved methods for CSF virus cultivation in different cell lines, CSF virus can be successfully accumulated *in vitro* in continuous porcine kidney cell line (IBRS), lamb testicle (LT) cell line, porcine kidney (PK) cell line, etc. [13–14].

Nevertheless, high-quality reproduction system development requires solving of the problems associated with the primary cell cultures used for virus-containing material scaling up for research purposes, or CSFV adaption to reproduction in continuous cell lines [11].

Use of the CSF virus preliminary adapted to growth in continuous cell culture lines could be helpful for partial overcoming of the said challenges and significantly simplifies cultivation process and standardizes the results of many diagnostic and experimental studies, but the number of adapted virus strains is limited [13, 15].

New CSF virus isolates are analyzed for their reproduction levels by cultivation in cell cultures that allows their biological properties to be determined and the virus-containing material to be accumulated for the virus genome extraction widely used for the determination of the agent relationship and possible disease spread routes [16].

The study was aimed at selection of the virus strains having high replication rates *in vitro* and testing of CSF virus strains for their reproduction properties in different cell cultures.

MATERIALS AND METHODS

The following CSF virus strains were used:

– "Sinlak" vaccine strain prepared from lapinized "K" strain through intermittent passages in PK-13, A_4C_2 cell cultures and adult rabbits (the strain was provided by the Laboratory for Porcine and Horned Livestock Diseases of the FGBI "ARRIAH");

– "CSF Amur 19-10/WB-12555" strain isolated from wild boar in the Amur Oblast in 2019;

– reference "Shi-Mynn" strain at 48th passage in pigs.

The following cell cultures obtained from the Cell Culture Unit of the FGBI "ARRIAH" Innovation Department were used for tests of different cell cultures for their susceptibility to CSF virus: primary cell cultures (PK – porcine kidney, PT – porcine testicle, LT – lamb testicle cell cultures) and continuous cell cultures (PEK – porcine embryo kidney, PSGK – Siberian ibex kidney, SSs – porcine spleen (a subculture, underwent more than 70 passages), IBRS – porcine kidney cell cultures).

The following medium was used as a minimal essential medium (MEM) for CSF virus cultivation in different cell cultures: Eagle-MEM prepared according to the FGBI "ARRIAH" procedure, supplemented with 10% fetal bovine serum and containing 50 μg/cm^3 of gentamycin, 2.5 mg/1,000 cm^3 of amphotericin B and 0.3 mg/cm^3 of glutamine.

Confluent and subconfluent cell monolayers were infected with CSF virus at multiplicity of infection of at least 0.1–1.0 $CCID_{50}$/cell.

The cell cultures were infected as follows:

1. Infection without adsorption – the virus was inoculated in culture flasks containing completely formed monolayer of 2–3-day-grown primary and continuous cell cultures.

2. Infection without adsorption – the cell culture was inoculated with the virus and left at 37 °C for 60 minutes, then, a required amount of the minimal essential medium was added to the culture flask. The flask with uninfected cell culture where the minimal essential medium was changed only served as control. The cell culture was daily observed for changes and detachment of the cells from the flask walls under CKX41 PhP FL US50 inverted laboratory microscope with binocular tube (Olympus, Japan).

3. Infection with minimal essential medium changing – Eagle-MEM supplemented with 10% normal porcine serum (freshly prepared and frozen serum was used for comparative testing) was used as a minimal essential medium. The medium was changed before cell culture infection by adding of 10 mL of Eagle-MEM containing 10% normal porcine serum.

The cell culture-containing plastic flasks with 25 cm^2 growth surface (T25) were incubated in thermostat or in CO_2 incubator at (37 ± 2) °C for 72–96 hours. Then, the following samples were taken from the culture flasks for testing with rtRT-PCR:

– from culture medium, at least 100 μL;

– from cell suspension, at least 100 μL.

Table 1
Results of rtRT-PCR tests of culture fluid and cell suspension samples collected on day 3–4 of CSF virus cultivation in primary and continuous cell cultures ($n = 3$)

CSF virus strain	Cell culture	Average Ct*	
		for cell culture	for cell suspension
"CSF Amur 19-10/WB-12555"	PK	16.4	18.27
	PSGK	18.25	20.01
	PEK	19.66	21.48
	SSs	15.52	17.02
"Shi-Mynn"	PK	17.83	19.11
	PSGK	17.63	19.64
	PEK	17.94	19.86
	SSs	15.85	17.19

* Average Ct value for 3 tested samples.
Real-time RT-PCR results interpreted in accordance with the rtRT-PCR kit manufacturer are as follows:
– positive result – Ct value is not higher than 33.0;
– inconclusive result – Ct value is higher than 33.0;
– negative result – Ct value is absent.

Table 2
Results of rtRT-PCR tests of culture fluid samples collected during CSF virus cultivation in primary and continuous cell cultures when the cell monolayer was infected with or without adsorption ($n = 3$)

CSF virus strain	Cell culture	Average Ct*	
		inoculation without adsorption	inoculation with adsorption
"CSF Amur 19-10/WB-12555"	SSs	16.27	20.91
	PK	16.22	16.26
	PEK	13.76	17.74
	PSGK	15.18	15.50
"Shi-Mynn"	SSs	10.78	10.82
	PK	12.97	14.37
	PEK	16.03	16.51
	PSGK	12.14	12.17

* Average Ct value for 3 tested samples.
Real-time RT-PCR results interpreted in accordance with the rtRT-PCR kit manufacturer are as follows:
– positive result – Ct value is not higher than 33.0;
– inconclusive result – Ct value is higher than 33.0;
– negative result – Ct value is absent.

The subsequent passaging was carried out by direct transfer of the virus-containing suspension to fresh cell culture or after thrice freezing-thawing of the culture fluid of the previous passage.

In case of absence of apparent CSFV-induced cytopathic effect the samples were tested for the virus and virus antigen with direct immunofluorescence test in accordance with the "Methodical Guidelines for classical swine fever virus isolation in different cell cultures followed by the virus identification with immunofluorescence test" [17], or for the virus RNA with rtRT-PCR in accordance with "Methodical Guidelines for classical swine fever virus isolation in primary cell cultures (PS, PBM, PK, PT, LT) followed by the virus identification with real-time polymerase chain reaction including detection using fluorescent hybridization probes" [18].

RESULTS AND DISCUSSION

Comparative analysis of virulent "CSF Amur 19-10/WB-12555" strain and reference "Shi-Mynn" strain as well as vaccine "Sinlak" strain accumulation levels was carried out for determination of CSF virus replication peculiarities in primary and continuous cell cultures.

The replication rate was estimated based on the time of maximum virus release in the minimal essential medium. For this purpose, culture fluid and cell monolayer samples collected on day 3–4 of cultivation were tested with rtRT-PCR (according to the test-kit manufacturer's instruction) providing that the highest cycle threshold (Ct) values correlated to the minimum CSF virus accumulation [19].

The test results given in Table 1 show that more than 90% of the virus-containing material were found in the culture fluid rather than in the cells on day 3–4 of the CSF virus cultivation. Maximum virus accumulation with the virus release in the minimal essential medium was observed in PK and SSs cell cultures.

At the next stage, two methods for cell infection: virus inoculation directly to the minimal essential medium and virus inoculation with adsorption on the cell monolayer for one hour were analyzed for their effectiveness. Accumulation levels were assessed with rtRT-PCR based on Ct values as in previous tests.

Based on the data given in Table 2 there were no significant differences in the virus accumulation in the cell culture when the virus-containing material was inoculated with adsorption on monolayer and directly to the minimal essential medium. The only one significant difference was observed for "CSF Amur 19-10/WB-12555" strain during its replication in SSs cell culture when Ct value was 20.91 in the cells infected with adsorption and 16.27 in the cells infected without adsorption.

Thus, analysis of the results of cultivation in four different cultures showed that effectiveness of the cell culture infection by CSF virus inoculated with adsorption on the cell monolayer was higher by 8.8% as compared to the infection by CSF virus inoculated directly to the minimal essential medium.

Ten percent inactivated normal porcine serum was added to the minimal essential medium to enhance the cell culture regenerative capacity and to increase the number of the cells attached to the culture flask walls. CSFV "Sinlak" vaccine strain was additionally used for comparative tests of the virulent CSFV strains.

Before the experiment and data analysis, "Sinlak" vaccine strain of CSF virus was subjected to three serial passages in primary PT and LT cell cultures to achieve desired Ct values (10–12) when it was tested with rtRT-PCR. Then, the virus was accumulated in the minimal essential medium supplemented with 10% inactivated normal porcine serum and parallelly in minimal essential medium supplemented with 10% fetal bovine serum for comparative analysis.

Analysis of the data given in Table 3 shows that CSF virus replication rate was the highest when the nutrient medium was changed and supplemented with 10% normal porcine serum. Significant decrease in Ct values indicative of increase in the virus titres was observed under the said conditions both for virulent and vaccine CSF virus strains.

Ct values detected with rtRT-PCR were found to be the lowest in primary PT and LT cell cultures (for the vaccine strain), that correlated with the higher virus accumulation in the said cell cultures. The said correlation was observed for both vaccine and virulent virus strains. Analysis of Ct values for the samples taken from PK and IBRS cell cultures allows us to conclude that these cultures are ineffective for the virus accumulation, therefore, CSF virus requires preliminary adaptation to these cell cultures.

Thus, PT cell culture was found to be 58% more effective for CSF virus accumulation (virulent strains and vaccine strain) as compared to LT, PK and IBRS cell cultures.

In subsequent experiments, the effectiveness of the freshly prepared and frozen normal pig sera use in the minimal essential medium changed during the cultivation was compared.

Test results showed that the highest Ct values were detected with rtRT-PCR when the frozen porcine serum was used. However, it cannot be excluded that freshly prepared porcine serum used in this experiment could contain a large number of anti-CSFV antibodies and, consequently, reduced the virus replication rate. Hence, porcine serum should be tested for anti-CSFV antibodies with ELISA prior to its adding to the minimal essential medium.

According to the results of the set of experiments, it was found that the CSF virus demonstrated the best reproductive properties in the primary PT cell culture, the average cycle threshold (Ct) value was 12.89 when the said cell culture was used.

Table 3
Results of rtRT-PCR tests of culture fluid samples collected during CSF virus cultivation in primary and continuous cell cultures with or without changing of minimal essential medium supplemented with 10% normal porcine serum (*n* = 3)

CSF virus strain	Cell culture	Average Ct*	
		without MEM changing	with MEM changing
"Sinlak"	PT	9.41	8.22
	LT	12.18	14.29
	PK	23.84	21.93
"CSF Amur 19-10/WB-12555"	PT	11.09	10.69
	PK	14.98	14.52
	IBRS	16.56	16.10
"Shi-Mynn"	PT	8.08	7.17
	PK	12.37	11.71
	IBRS	13.83	13.74

* Average Ct value for 3 tested samples.
Real-time RT-PCR results interpreted in accordance with the rtRT-PCR kit manufacturer are as follows:
– positive result – Ct value is not higher than 33.0;
– inconclusive result – Ct value is higher than 33.0;
– negative result – Ct value is absent.

Table 4
Results of rtRT-PCR tests of culture fluid samples collected during CSF virus cultivation in primary porcine testicle and lamb testicle cell cultures in the minimal essential medium supplemented with freshly prepared or frozen 10% porcine serum (*n* = 3)

CSF virus strain	Cell culture	Average Ct*	
		when freshly prepared porcine serum was added	when frozen porcine serum was added
"Sinlak"	LT	17.57	13.26
	PT	16.48	11.16
"CSF Amur 19-10/WB-12555"	PT	15.63	14.71
"Shi-Mynn"	PT	15.09	12.08

* Average Ct value for 3 tested samples.
Real-time RT-PCR results interpreted in accordance with the rtRT-PCR kit manufacturer are as follows:
– positive result – Ct value is not higher than 33.0;
– inconclusive result – Ct value is higher than 33.0;
– negative result – Ct value is absent.

CONCLUSION

Since diagnosticum development requires accumulation of CSFV antigen and genome in large amounts, the main goal of the study was to achieve high CSF virus reproduction in cell culture. Maximum CSFV accumulation with the virus release in the minimal essential medium was registered on day 3–4 of cultivation. The virus inoculation of the cell culture with adsorption was found to be ineffective. It is reasonable to add 10% porcine CSFV antibody-negative sera instead of fetal bovine serum.

Based on the tests of vaccine and virulent CSFV strains for their reproductive properties in primary and continuous cell cultures, the following strains are selected for further development of diagnostica: the "Sinlak" vaccine strain that demonstrated effective accumulation in PT and LT cell cultures when the minimal essential medium was supplemented with 10% normal porcine serum and virulent "CSF Amur 19-10/WB-12555" strain originating from the isolate recovered from a wild boar in the Amur Oblast of the Russian Federation in 2019 that better accumulated in PT cell cultures.

Genetic analysis of these CSF virus variants is required for determination of unique genetic markers allowing differentiation between CSFV strains and isolates for further tests of the selected strains for their use for diagnostic purposes.

In the above context, it should be noted that the development of DIVA strategy as an improved tool that can be successfully used in CSF-enzootic countries for CSFV circulation monitoring as well as in CSF-affected countries for minimization of the virus spread and economic losses in case of the disease outbreak is critical for CSF eradication and spread control.

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Influence of bovine blood serum on growth properties of nutrient media for *Mycoplasma gallisepticum* and *Mycoplasma synoviae* cultivation

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SUMMARY

The growth properties of the nutrient medium for the cultivation of pathogenic mycoplasmas depend on the type of blood serum it is supplemented with. Comparative tests of two cell-free nutrient media supplemented with bovine and porcine blood sera for the cultivation of strains "S6" *Mycoplasma gallisepticum* and "WVU 1853" *Mycoplasma synoviae* were performed. Growth properties of the tested nutrient media were assessed by determining the activity of the resulting biomass in the hemagglutination and agglutination assays, as well as by determining the concentration of viable cells after the 9th passage. It has been shown that a cell-free nutrient medium supplemented with the porcine blood serum is optimal for the cultivation of pathogenic mycoplasma species causing infectious diseases in birds. The hemagglutinating activity of the *Mycoplasma gallisepticum* culture reached 5 HAU $\log_2$ after 72 hours of cultivation, the agglutinating activity of *Mycoplasma synoviae* reached 5 AU $\log_2$ during the 88-hour incubation period, the concentration of viable cells of both strains was 10^6 CFU/cm^3. The low growth properties of the medium prepared with the addition of bovine blood serum are most likely associated with its biochemical composition, which contains 5–20 times more provitamin A than the porcine blood serum, and high density lipoprotein cholesterol. On the contrary, in the porcine blood serum, most of the lipoproteins have a low density, containing a large amount of fatty acids and cholesterol, which are the main structural elements of mycoplasma cells. The obtained test results are of practical value and can be used in the technology of cultivation of pathogenic species of avian mycoplasmas in the production of diagnostic and preventive tools.

Keywords: *Mycoplasma gallisepticum*, *Mycoplasma synoviae*, nutrient medium, cultivation, blood serum

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Влияние сыворотки крови крупного рогатого скота на ростовые свойства питательной среды для культивирования *Mycoplasma gallisepticum* и *Mycoplasma synoviae*

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РЕЗЮМЕ

Ростовые свойства питательных сред для культивирования патогенных микоплазм зависят от вида сыворотки крови в их составе. Проведены сравнительные испытания двух бесклеточных питательных сред с добавлением сывороток крови свиней и крупного рогатого скота для культивирования штаммов «S6» *Mycoplasma gallisepticum* и «WVU 1853» *Mycoplasma synoviae*. Изучение ростовых свойств испытуемых питательных сред проводили путем определения активности полученной биомассы в реакции гемагглютинации и реакции агглютинации, а также оценки концентрации жизнеспособных клеток после 9-го пассажа культивирования. Показано, что бесклеточная питательная среда с сывороткой крови свиней является оптимальной для культивирования патогенных видов микоплазм, вызывающих инфекционные заболевания у птиц. Гемагглютинирующая активность культуры *Mycoplasma gallisepticum* достигала 5 ГАЕ $\log_2$ после 72 ч культивирования, агглютинирующая активность *Mycoplasma synoviae* – 5 АЕ $\log_2$ за 88-часовой период инкубации, концентрация жизнеспособных клеток обоих штаммов была на уровне 10^6 КОЕ/см^3. Низкие ростовые свойства среды, приготовленной с добавлением сыворотки крови крупного рогатого скота, вероятнее всего, связаны с ее биохимическим составом, которая содержит в 5–20 раз больше провитамина А, нежели сыворотка крови свиней, а холестерин в основном представлен липопротеинами высокой плотности. Напротив, в сыворотке крови свиней большая часть липопротеинов имеет низкую плотность, содержащих большое количество жирных кислот и холестерина, которые и являются основными структурными элементами клеток микоплазм. Полученные результаты исследований имеют практическую ценность и могут быть использованы в технологии культивирования патогенных видов микоплазм птиц при производстве средств диагностики и профилактики.

Ключевые слова: *Mycoplasma gallisepticum*, *Mycoplasma synoviae*, питательная среда, культивирование, сыворотка крови

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INTRODUCTION

Mycoplasmas are bacteria, the key difference of which from other representatives of prokaryotes is the absence of a cell wall, which explains their resistance to a number of antibacterial drugs. The variety of forms of these microorganisms includes spherical, disk-shaped, rod-shaped and filamentous structures, varying in size from 0.1 to 1.5 microns. Another feature of mycoplasmas is that during growth on nutrient media and cell division, their size is less than the theoretical limit of self-reproduction. Mycoplasmas reproduce by budding, binary fission, fragmentation, divide by cells of unequal size, as a result of which one of the newly formed cells may not be viable. Many mycoplasma species are difficult-to-cultivate microorganisms and are poorly adapted to nutrient media, which creates certain difficulties in the technology for the production of diagnostic systems and vaccine preparations [1, 2].

In the process of growth, they need amino acids (arginine, isoleucine, methionine, phenylalanine, asparagine). A need for bile salts and fatty acids has been noted. One of the media that can ensure the growth of many types of mollicutes is modified Frey's medium, which includes PPLO-broth, dextrose, pig blood serum, β-nicotinamide adenine dinucleotide and L-cysteine hydrochloride. This medium is a source of amino acids, carbohydrates, and various vitamins necessary for the growth of mycoplasmas [2, 3]. The blood serum for the nutrient medium is obtained mainly from pigs or horses, since the blood serum of other animals can not only slow down growth, but rather completely inhibit it. Blood serum, as a growth stimulator of mycoplasmas, is an important component of the medium, without which cultivation on cell-free nutrient media becomes impossible.

The study of the possibility of using blood sera from different animal species as part of nutrient media for mycoplasmas can be a positive experience in the issue of their laboratory or industrial cultivation.

When it comes to oxygen requirements, mycoplasmas belong either to strict aerobes or to obligate anaerobes. Their growth lasts from 3 to 7 days at first inoculations, then in the process of reinoculation they can grow significantly faster.

The cultivation of these microorganisms is an important link in the creation of diagnostic tools, specific prophylaxis, which is an alternative to antibiotic therapy in the eradication of infections of mycoplasma etiology on industrial poultry farms [4].

The problem of mycoplasmoses arose as a result of the introduction of intensive poultry rearing methods at establishments, which in turn led to their wide spread among poultry [5, 6]. Infections such as respiratory mycoplasmosis and infectious synovitis affect chickens, turkeys, pigeons, quails and partridges. Clinical manifestations of respiratory mycoplasmosis (causative agent – *Mycoplasma gallisepticum*) are respiratory system disorders (rhinitis, laryngitis, aerosacculitis, pneumonia), conjunctivitis, sinusitis, bursitis, anemia, tendovaginitis are distinguished among systemic disorders [7–9].

The economic damage caused by these diseases consists of a decrease in the laying productivity of poultry, slow growth, and a decrease in the egg hatchability. Moreover, an infection caused by *Mycoplasma synoviae* is characterized by a decrease in the quality of the eggshell – a manifestation of the vitreous apex syndrome [10]. The immunosuppressive properties of mycoplasmas make ineffective measures for the specific prevention of other economically significant diseases, preventing the development of an immune response during live vaccine administration [11–13].

The study of the influence of essential components in the medium affecting mycoplasma growth, in this case in the bovine blood serum, is a serious challenge in mycoplasmology when creating means for diagnosing and preventing the infections in question.

MATERIALS AND METHODS

The "WVU 1853" *Mycoplasma synoviae* and "S6" *Mycoplasma gallisepticum* strains from the FGBI "VGNKI" (Moscow) were used in the study.

Mycoplasma gallisepticum and *Mycoplasma synoviae* were cultivated on a liquid and dense modified Frey's nutrient medium, which included distilled water, a medium for the cultivation of pleuropneumonia-like organisms (PPLO broth base), yeast extract, glucose, thallium acetate, and bovine as well as porcine blood serum [14]. Previous studies have shown that a 12% concentration of porcine blood serum in the medium ensures optimal growth of *Mycoplasma gallisepticum* and *Mycoplasma synoviae*, so the concentration of this component was not changed during the experiment. When growing *Mycoplasma synoviae*, *β*-nicotinamide adenine dinucleotide (1% solution) and L-cysteine hydrochloride (1% solution) were also added to the nutrient medium as a V-growth factor. Phenol red served as an indicator of biomass growth control. Since mycoplasmas are sensitive to the acidity of the medium, the acid-base balance during the preparation of the medium was adjusted to pH 7.8–8.0. To prevent the growth of foreign microorganisms, antibiotics (benzylpenicillin sodium salt) and thallium acetate (10% solution) were added to the medium [15].

The cultivation of mycoplasmas was carried out in a thermostat at a temperature of (37.5 ± 0.5) °C. The cultivation time depends on the type of mycoplasma. Thus, the optimal duration of *Mycoplasma gallisepticum* cultivation ranged from 48 to 96 hours, and *Mycoplasma synoviae* – from 3 to 7 days. The growth of biomass was monitored by the change in the color of the indicator and the transparency of the nutrient medium. During growth, the medium changed color from red-brown to yellow-brown and became slightly cloudy or opalescent. The growth of *Mycoplasma synoviae* was accompanied by the formation of an oil film on the surface of the medium.

The concentration of living cells of mycoplasmas was determined by titration on dense nutrient media in Petri dishes. For this, 10-fold dilutions were prepared for the culture of *Mycoplasma gallisepticum* and 5-fold dilutions for the culture of *Mycoplasma synoviae*, which were incubated for 5 days at a temperature of (37.5 ± 0.5) °C. Then the dishes were examined under a Micros MC 50 X microscope (Austria) at 200× magnification to detect characteristic colonies that looked like "fried eggs". The arithmetic mean of the colonies in several non-overlapping fields of view corresponds to the number of colony-forming units (CFU).

The calculation of CFU in the field of view of the microscope was carried out according to the formula:

$$T = N \times 10^{d} \times K / V,$$

where T – CFU titre in 1 cm^3;

N – arithmetic mean number of colonies in one field of view of the microscope;

d – culture dilution rate;

K – a coefficient equal to the ratio of the dish area to the area of the field of view of the microscope (the area of the field of view of the microscope was determined using a measuring glass);

V – volume of culture biomass added.

When evaluating the growth properties of a cell-free nutrient medium, attention was also paid to the determination of the hemagglutinating activity of *Mycoplasma gallisepticum* and the agglutinating activity of *Mycoplasma synoviae* in the hemagglutination and agglutination reactions, respectively to solid nutrient medium [16, 17].

RESULTS AND DISCUSSION

The main criterion in the selection of blood serum for the preparation of a nutrient medium is to obtain the

Table 1
Growth properties of the cell-free nutrient medium supplemented with the bovine blood serum for *Mycoplasma gallisepticum* cultivation

Cell-free nutrient medium samples	Cattle blood serum contents (%)	Biomass growth rate (h), passage 1–3	Biomass growth rate (h), passage 1–3 after cloning	Biomass growth rate (h), passage 1–3 after recloning	Culture activity at passage 9 in HA test (HAU $\log_2$)	Growth properties at passage 9 (CFU/cm^3)
1	12	118–120	115–120	94–96	2.0	$10^{3.5}$
2	15	118–120	115–120	94–96	2.5	10^{6}
3	20	117–120	114–120	92–94	3.0	10^{5}
4	25	115–120	112–120	90–94	3.0	10^{5}
Control (cell-free nutrient medium with pig serum)	12	72–78	68–72	68–72	5.0	10^{6}

maximum amount of biomass of cultivated cultures with the highest possible hemagglutinating (for *Mycoplasma gallisepticum*) and agglutinating (for *Mycoplasma synoviae*) activity.

During the experiment, 9 passages and 2 cloning procedures of both cultures were carried out at the 3rd and 6th passages. The cultures after the last passage were examined in hemagglutination and agglutination reactions to establish activity and growth properties after adaptation to a nutrient medium; at the same time passages in the control cell-free nutrient medium were performed (with porcine blood serum) to compare the results.

The criterion for selecting the type of blood serum for the preparation of the nutrient medium was based on the calculation of CFU for *Mycoplasma gallisepticum* and *Mycoplasma synoviae* under microscope on solid nutrient medium. The results of the experiment are presented in Table 1 and 2.

It was established that the cell-free nutrient medium supplemented with the bovine blood serum at a concentration of 12 to 25% ensured the growth and accumulation of *Mycoplasma gallisepticum* over 3 passages in the range from 115 to 120 hours. At the same time, the concentration of bovine blood serum in the medium did not significantly affect its growth properties. When using porcine blood serum at a concentration of 12% in the cell-free nutrient medium the cultivation time was reduced by 40–45 hours already at passage 1, which is an important factor in the technology for the production of diagnostics and prevention of infectious diseases of birds of mycoplasmal etiology.

To obtain an axenic culture of the microorganism at passage 3, cloning was performed on Frey's dense nutrient medium with thallium acetate. The use of the *Mycoplasma gallisepticum* clone enhances the adaptation of the microorganism to such new conditions as changing the composition of the culture medium, which in turn reduces the cultivation time and increases the concentration of mycoplasma cells in the biomass volume. Thus, after repeated cloning at the passage 3, the growth time significantly decreased from 120 to 96 hours.

When evaluating the hemagglutinating properties of *Mycoplasma gallisepticum* culture in HA test, it was found that after the 9th passage, the activity of the culture grown on the medium supplemented with bovine blood serum ranged from 2.0 to 3.0 $\log_2$, which is a relatively low indicator compared to the growth of the culture on the control medium with the porcine blood serum, where the HAU was 5.0 $\log_2$.

The calculation of living cells in 1 cm^3 of the nutrient medium showed that their lowest concentration ($10^{3.5}$ CFU/cm^3) was observed when using a medium containing 12% of bovine blood serum, while when using porcine blood serum (12%), the concentration of mycoplasma cells was the highest and amounted to 10^6 CFU/cm^3. An increase in the concentration of cattle blood serum in the medium from 15 to 25% had a positive effect on its growth properties, while the CFU titer was in the range of 10^5–10^6 per 1 cm^3. However, the quality of the resulting antigen is determined not only by the concentration of cells in the volume of the medium, but by a set of indicators, including growth time, hemagglutinating and agglutinating activity, CFU. Thus, the porcine blood serum in Frey's nutrient medium ensures the maximum accumulation of the *Mycoplasma gallisepticum* culture with a minimum cultivation time.

Figure 1 shows micrographs of *Mycoplasma gallisepticum* colonies grown on a nutrient medium with bovine blood serum. The colonies of the pathogen in the field

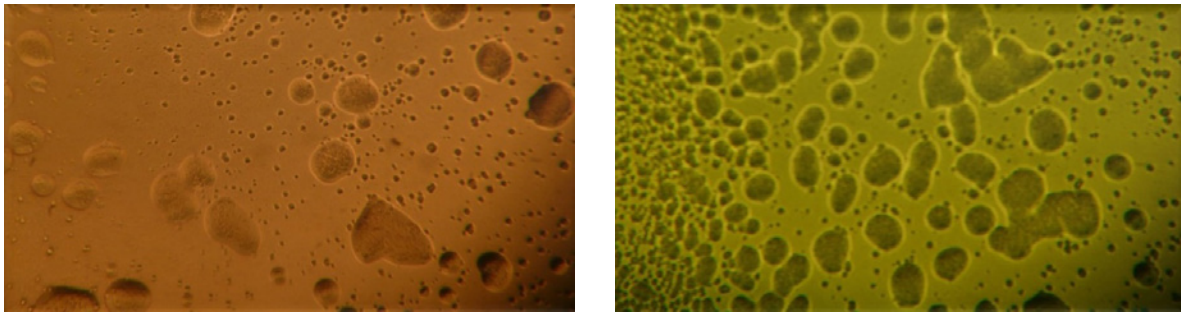

Fig. 1. Mycoplasma gallisepticum colonies, grown on the cell-free nutrient medium supplemented with the bovine blood serum (magnification 200×)

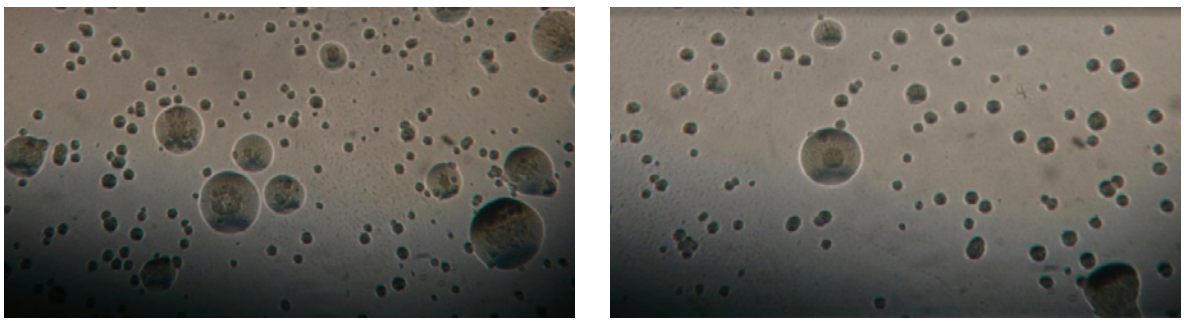

Fig. 2. Mycoplasma gallisepticum colonies, grown on the cell-free nutrient medium supplemented with the porcine blood serum (magnification 200×)

Table 2
Growth properties of the cell-free nutrient medium supplemented with the bovine blood serum for *Mycoplasma synoviae* cultivation

Cell-free nutrient medium samples	Cattle blood serum contents (%)	Biomass growth rate (h), passage 1–3	Biomass growth rate (h), passage 1–3 after cloning	Biomass growth rate (h), passage 1–3 after recloning	Culture activity at passage 9 in HA test (HAU log_2)	Growth properties at passage 9 (CFU/cm^3)
1	12	140–144	115–120	94–96	2.0	10^3
2	15	140–144	115–120	94–96	2.5	10^3
3	20	138–140	114–120	92–94	3.0	10^4
4	25	138–140	112–120	90–94	3.0	10^4
Control (cell-free nutrient medium with pig serum)	12	78–96	78–88	78–88	5.0	10^6

of view of the microscope are unevenly distributed, their morphology is polymorphic, there are oval, pear-shaped, poorly formed colonies of different sizes, the center is not pronounced or occupies most of the colony.

Figure 2 shows colonies of *Mycoplasma gallisepticum* grown on a nutrient medium supplemented with the porcine blood serum. Despite the colonies varying in size, round shapes with smooth edges and a distinctly formed and denser optical center predominate, which gives them the "fried egg" appearance characteristic of mycoplasmas.

The results of studies on the growth properties of the cell-free nutrient medium, which includes bovine blood serum, for growing *Mycoplasma synoviae* showed that the cultivation time is at least 140 hours at a serum concentration in the medium of 12 and 15% and 138 hours at a concentration of 20–25% (Table 2). When accumulating the biomass of *Mycoplasma synoviae*, it should also be taken into account that this type of mycoplasma belongs to difficult-to-cultivate microorganisms and has a lower adaptive capacity compared to *Mycoplasma gallisepticum*.

Similar to the results obtained in the study of the growth properties of the cell-free nutrient medium for the cultivation of *Mycoplasma gallisepticum*, when using the *Mycoplasma synoviae* clone, a reduction in the cultivation time to 96 hours was observed at passages 7–9. The agglutinating activity of *Mycoplasma synoviae* culture and the concentration of viable cells increased with an increase in the concentration of bovine blood serum in the medium from 12 to 20% and were in the range from 2.0 to 3.0 log_2 and 10^3–10^4 CFU/cm^3, respectively. At the same time, the activity of the culture grown on Frey's medium with porcine blood serum (12%) was 5.0 log_2, and the CFU value was 10^6 per 1 cm^3, which reliably indicates the clear advantages of using porcine blood serum in the cell-free nutrient medium for cultivating *Mycoplasma synoviae*.

Figure 3 shows the result of cultivating *Mycoplasma synoviae* on dense cell-free nutrient medium supplemented with the bovine blood serum (day 6 of culturing). The grown colonies are small in size, their diameter does not exceed 0.1 mm, and there is no optically dense center in their structure. The micrograph of Figure 4 shows colonies of *Mycoplasma synoviae* grown on a medium with porcine blood serum (6 days after seeding). Colonies are characterized by the appearance of "fried eggs", they are larger – up to 0.2 mm in diameter.

Thus, the results of the tests performed indicate that the growth properties of nutrient media for the cultivation of pathogenic mycoplasmas depend on the type of blood serum in their composition. It was noted that an increase in its concentration stimulated the growth of biomass with a reduction in the cultivation time. The use of porcine blood serum in the composition of the nutrient medium

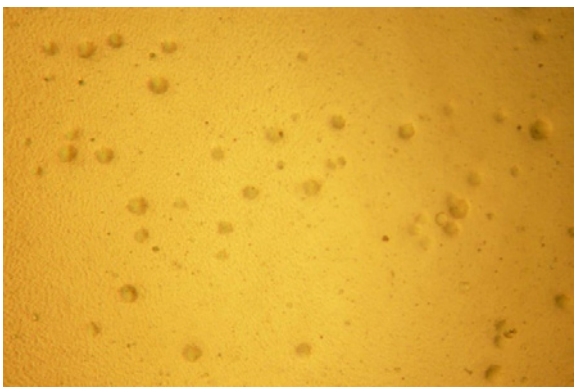

Fig. 3. Mycoplasma synoviae grown on the cell-free nutrient medium supplemented with bovine blood serum (magnification 200×)

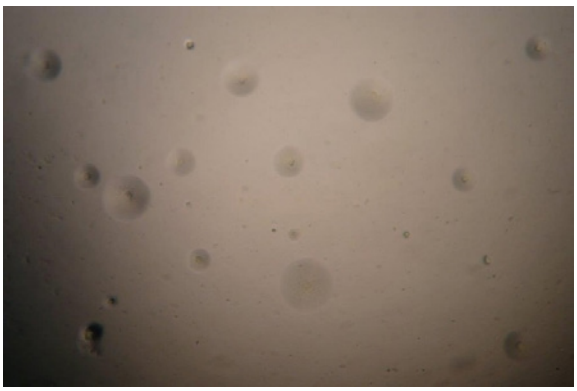

Fig. 4. Mycoplasma synoviae grown on the cell-free nutrient medium supplemented with porcine blood serum (magnification 200×)

at a concentration of 12% provided a more active biomass both in terms of the concentration of viable cells and the hemagglutinating and agglutinating activity of the culture with a minimum cultivation time of 78–96 hours.

To establish the reason for the low growth properties of the nutrient medium with the addition of cattle blood serum, a comparative analysis of some biochemical parameters of bovine and porcine blood serum was performed (Table 3) [18].

Despite the fact that the bovine and porcine blood serum contains a comparable amount of cholesterol, cattle have a large amount of high-density lipoproteins, characterized by a significantly low content of cholesterol and phospholipids [19]. Thus, serum with a high content of these lipoproteins may not meet the needs of mycoplasmas in fatty acids and cholesterol. On the contrary, the blood serum of pigs contains mainly low-density lipoproteins, which actively carry cholesterol and fatty acids, which can partially precipitate without any external influence. It is known that the causative agents of mycoplasmoses need these components, and the porcine blood serum makes up for the need for these compounds when cultivating mycoplasmas on the free-cell nutrient medium [14, 20].

Also, from the presented data, it can be seen that the carotene content in the bovine blood serum exceeds its concentration in the porcine blood serum from 5 to 20 times, and retinol – 3 times. Given that the components in question are the strongest antioxidants, they can slow down oxidative processes in cells, thereby increasing the interphase period or slowing down the process of accumulating resources for normal cell division, thereby initiating the appearance of non-viable mycoplasma cells. The close to zero carotene content in the porcine blood serum eliminates the risks associated with its potential impact on the growth and reproduction of mollicutes.

The results of the study indicate that the porcine blood serum is a significant growth component for the cultivation of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* and provides a more active biomass in the medium.

CONCLUSION

As a result of the research, it was found that the cell-free nutrient medium supplemented with porcine blood serum has the most suitable growth characteristics for the optimal growth of *Mycoplasma synoviae* and *Mycoplasma gallisepticum* culture. The medium supplemented with bovine serum can also be used for the cultivation of these species of mollicutes, but it does not provide the same growth properties as the cell-free nutrient medium with porcine blood serum.

The low growth properties of the medium prepared using bovine blood serum are most likely associated with its biochemical composition, which contains 5–20 times more provitamin A than porcine blood serum, and cholesterol is mainly represented by high density lipoproteins. On the contrary, in the porcine blood serum, most of the lipoproteins have a low density, containing a large amount of fatty acids and cholesterol, which are the main structural elements of mycoplasma cells, in particular for the cytoplasmic membrane, which ensure its fluidity. The high content of carotene in the blood serum of cattle can increase the period of interphase during the reproduction of mycoplasmas.

Table 3
Biochemical parameters of the bovine and porcine blood sera

Parameters	Bovine blood serum	Porcine blood serum
Serum cholesterol:		
mg/100 mL	50–170	60–110
mmol/L	1.30–4.42	1.56–2.86
Serum carotene:		
μg/100 mL	500–2,000	0–10
mg/L	5.0–20.0	0–0.1
Serum Vitamin A:		
μg/100 mL	30–90	10–35
μmol/L	1.05–3.14	0.35–1.22

Twelve percent concentration of porcine blood serum in the cell-free nutrient medium is optimal for preparing a culture with high biological activity with a short cultivation time (72–96 hours).

Adaptation of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* to the cell-free nutrient medium supplemented with the bovine blood serum prior to passage 9 did not allow preparing a biomass with an activity similar to that of the medium with porcine blood serum, which should be taken into account when cultivating these species of mollicutes on a large scale.

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Cryopreservation of primary trypsinized fibroblast cells of chicken embryos using various cryoprotectants

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SUMMARY
Cryopreservation is the optimal way to store cells at ultra-low temperatures. Cryoprotectants are added to cell culture suspension to reduce cell death due to exposure to low temperatures. Cryoprotective media contain combinations of various cryoprotectants. Ethylene glycol, glycerin, dimethyl sulfoxide, sucrose, dextran, propylene glycol, albumin, polyvinylpyrrolidone and blood serum can be used as cryoprotectants. For cryopreservation it is necessary to select a cryoprotectant that ensures the highest survival of cells after storage and thawing. The paper presents the results of experiments on comparing the effectiveness of dimethyl sulfoxide, ethylene glycol and glycerin in cryopreservation of primary trypsinized chicken embryo fibroblasts. As a result of cell suspension equillibration (incubation at room temperature) with serum and the specified cryoprotectants at different concentrations, the suspension variants containing different cryoprotectant and serum ratios were selected for freezing. Previously, it was found that after 12 months of observation, when using dimethyl sulfoxide as a cryoprotectant, the largest number of surviving cells (46%) was observed in a suspension containing 20% fetal serum and 10% dimethyl sulfoxide. The amount of surviving cells if 10% fetal serum and 5% ethylene glycol were included in the cryoprotective mixture was slightly lower and amounted to 36% after 12 months of observation. Glycerin is shown to have weak protective properties as regards chicken embryo fibroblast cells. After 8 months of storage, the amount of surviving cells in a suspension containing 10% serum and 5% glycerin was 22%, no live cells were found in this mixture if stored longer. The proliferative properties of cells and their sensitivity to viruses remained within the 12 months of the experiment.

Keywords: cryopreservation, chicken embryo fibroblast cells, cryoprotectants

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УДК 619:576.3:57.043

Криоконсервирование первично трипсинизированных клеток фибробластов эмбрионов кур с использованием разных криопротекторов

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РЕЗЮМЕ

Криоконсервирование является оптимальным способом хранения клеток при сверхнизких температурах. Для уменьшения гибели клеток от воздействия низких температур к суспензии клеточной культуры добавляют криопротекторы. Сочетания различных криопротекторов образуют криозащитные среды. В качестве криопротекторов могут быть применены этиленгликоль, глицерин, диметилсульфоксид, сахароза, декстран, пропиленгликоль, альбумин, поливинилпирролидон и сыворотка крови. При проведении работ по криоконсервированию необходимо подобрать криопротектор, обеспечивающий наибольшую выживаемость клеток после хранения и оттаивания. В данной статье представлены результаты экспериментов по сравнению эффективности диметилсульфоксида, этиленгликоля и глицерина при криоконсервировании первично трипсинизированных клеток фибробластов эмбрионов кур. В результате эквилибрации клеточной суспензии (инкубирования при комнатной температуре) с сывороткой и указанными криопротекторами разных концентраций для замораживания были выбраны варианты суспензий, содержащие различные соотношения криопротекторов и сыворотки. Ранее было установлено, что по истечении 12 месяцев наблюдения при использовании в качестве криопротектора диметилсульфоксида наибольшее количество выживших клеток (46%) наблюдалось в суспензии, содержащей 20% фетальной сыворотки и 10% диметилсульфоксида. Количество выживших клеток при наличии в составе криозащитной смеси 10% фетальной сыворотки и 5% этиленгликоля было несколько ниже и составило 36% по истечении 12 месяцев наблюдения. Было показано, что глицерин обладает слабыми протективными свойствами по отношению к клеткам фибробластов эмбрионов кур. Спустя 8 месяцев хранения количество выживших клеток в суспензии, содержащей 10% сыворотки и 5% глицерина, составило 22%, при последующем хранении живых клеток в данной смеси не выявляли. Пролиферативные свойства клеток и чувствительность их к вирусам сохранялись на протяжении 12 месяцев эксперимента.

Ключевые слова: криоконсервирование, клетки фибробластов эмбрионов кур, криопротекторы

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INTRODUCTION

Cryopreservation is widely used for long-term storage of live cells, tissues, organelles, organs and whole organisms at ultra-low temperatures for a long period of time (from several months to several years). To be more specific, cryopreservation is the optimal way to store cell cultures of coelenterates [1] and other live organisms, mammal sperm and embryos, etc. A number of works have also been carried out to improve methods of freezing fish reproductive material [2, 3]. In recent years, cryopreservation methods of human cells and tissues were developed and updated. Thus, the manual cryopreservation method for erythrocytes was improved at the Federal State Budget Institution "Russian Research Institute of Hematology and Transfusiology of the Federal Medical and Biological Agency" (St. Petersburg) [4]. Japanese researchers developed a method for cryopreservation of human corneal endothelial cell culture [5].

Developments on cryopreservation of animal cell cultures and study of the biological properties of frozen cells are also important. Researchers of FGBI "ARRIAH" (Vladimir) developed methods for cryopreservation of continuous BHK-21 cell culture, primary trypsinized culture of chicken embryo fibroblast cells (CEF) containing Marek's disease virus, and primary culture of porcine bone marrow cells [6–8]. Specialists of the Federal State Budgetary Scientific Institution "Federal Center for Toxicological, Radiation, and Biological Safety" (Kazan) conducted studies of the biological properties of the transplanted bovine lung embryonic cell culture (LEK) subjected to long-term cryopreservation [9]. In addition, the results of the study on cryopreservation of broiler chicken embryo fibroblast cells were published [10].

An important aspect of cell culture cryopreservation is optimal selection of a cryoprotectant – a substance that protects cells from the freezing damage [6, 11]. There are two types of cryoprotectants: penetrating ones that easily penetrate into cells (dimethyl sulfoxide – DMSO, glycerin, ethylene glycol, propylene glycol), and non-penetrating ones that ensure external protective environment for cells (sucrose, dextran, albumin, polyvinylpyrrolidone) [11–14]. A cryoprotectant is often used in combination with stabilizers, commonly with blood sera [6–8, 13]. It is known about the use of a mixture of embryonic serum, DMSO and gold nanoparticles as a cryoprotective medium for human fibroblast cells [15]. Development of cryopreservation methods for different types of cell cultures suggested a high degree of cell survival (primary cells – about 58%, continuous cells – more than 90%) after freezing [2, 12]. There are a lot of combinations of preservation media. A mixture of cell suspension, serum and cryoprotectant is most commonly used in practice [6–8].

CEF cell culture is of great practical importance in virology and biological production, therefore, searching new, optimal combinations of preservation media during cell freezing remains relevant. Previously, some works on

selecting the optimal combination of DMSO and serum for cryopreservation of primary trypsinized CEF cells were carried out [16], however, no similar studies were conducted for other cryoprotectants.

The aim of this paper was to study the protective properties of various cryoprotectants during cryopreservation of primary trypsinized CEF cells and compare their effectiveness. At the same time, such indicators as cell survival after storage at ultra-low temperatures, proliferative properties and cell susceptibility to viruses after freezing were evaluated.

Based on the aim, the following objectives were set: selection of cryoprotectant-stabilizer combination that is not toxic to cells; study of cryoprotectant protective properties for freezing CEF cell suspension, as well as study of cell culture proliferative properties and sensitivity to viruses after freezing.

MATERIALS AND METHODS

Selection of the optimal combination of serum and cryoprotectants added to the cell suspension. CEF cell suspension for freezing was prepared according to previously developed methods [16, 17]. DMSO (Carl Roth, Germany), glycerin (JSC "Himreactiv", Russia) and ethylene glycol (JSC "Himreactiv", Russia) were used as cryoprotectants. Combinations of mixtures containing the cell suspension, the specified cryoprotectants and the fetal serum (BioClot, Brazil) used as a stabilizer were prepared. Table 1 shows variants of cryoprotective mixtures, including different combinations of cryoprotectants and fetal serum (as a percentage in the suspension volume).

Afterwards the cell mixture was equilibrated (incubated at a room temperature) with cryoprotectants and a serum, the suspensions prepared for cryopreservation were subsequently placed for storage.

Assessment of cell viability after thawing. This step was carried out according to the previously developed method [16].

Determination of cryopreserved CEF cell sensitivity to viruses. The cell cultures under study were infected with the reference strain of the avian leukosis virus ALV-J ADOL-HCI-P7 after 3rd passage (Avian Disease and Oncology Laboratory, USA). After the incubation period, the virus accumulation was determined by the enzyme immunoassay (ELISA) based on the concentration of virus-specific protein p27 using a commercial ProFLOK ALV Plus Ag kit (Zoetis, USA) according to the manufacturer's instructions [16].

Table 1
Samples of CEF cell suspension supplemented with cryoprotective agents

	10% serum				20% serum			
DMSO content	5%	10%	15%	20%	5%	10%	15%	20%
Glycerin content	5%	10%	15%	20%	5%	10%	15%	20%
Ethylene glycol content	5%	10%	15%	20%	5%	10%	15%	20%

RESULTS AND DISCUSSION

The work on comparing the effectiveness of various cryoprotectants and optimizing the conditions for CEF cell cryopreservation included the following steps: selection of optimal concentrations of cryoprotectants in a preservation mixture; study of viability and proliferative properties of cells after storage at minus 150 °C for 12 months.

To perform the first step, the equilibration of CEF cell suspensions containing the above specified combinations of cryoprotective agents was carried out (Table 1). The cell concentration in the initial cell suspension was $(1.10 \pm 0.01) \times 10^7$ cells/cm^3. The test results are shown in Table 2.

The test results showed that the highest cell survival (70–100%) was observed for the following variants of cryopreservation suspensions:

1) 10% serum and 5% DMSO (97% of surviving cells);
2) 10% serum and 10% DMSO (97% of surviving cells);
3) 10% serum and 15% DMSO (100% surviving cells);
4) 20% serum and 5% DMSO (97% of surviving cells);
5) 20% serum and 10% DMSO (100% surviving cells);
6) 20% serum and 15% DMSO (89% of surviving cells);
7) 10% serum and 5% glycerin (90% of surviving cells);
8) 10% serum and 5% ethylene glycol (74% of surviving cells).

Table 2
Cell concentration in suspensions for cryopreservation after equilibration, cells/cm^3 ($n = 3$)

10% serum				
Cryoprotectants	5%	10%	15%	20%
DMSO	**$(1.07 \pm 0.06) \times 10^7$**	**$(1.07 \pm 0.06) \times 10^7$**	**$(1.10 \pm 0.00) \times 10^7$**	$(6.67 \pm 0.30) \times 10^6$
Glycerin	**$(1.00 \pm 0.10) \times 10^7$**	$(7.20 \pm 0.26) \times 10^6$	$(6.23 \pm 0.32) \times 10^6$	$(3.26 \pm 0.38) \times 10^6$
Ethylene glycol	**$(8.17 \pm 0.15) \times 10^6$**	$(7.60 \pm 0.30) \times 10^6$	$(7.60 \pm 0.30) \times 10^6$	$(6.70 \pm 0.17) \times 10^6$
20% serum				
	5%	10%	15%	20%
DMSO	**$(1.07 \pm 0.06) \times 10^7$**	**$(1.10 \pm 0.00) \times 10^7$**	**$(0.98 \pm 0.10) \times 10^7$**	$(7.20 \pm 0.46) \times 10^6$
Glycerin	$(8.17 \pm 0.29) \times 10^6$	$(5.63 \pm 0.47) \times 10^6$	$(3.87 \pm 0.30) \times 10^6$	$(2.60 \pm 0.36) \times 10^6$
Ethylene glycol	$(6.90 \pm 0.00) \times 10^6$	$(6.80 \pm 0.17) \times 10^6$	$(6.03 \pm 0.15) \times 10^6$	$(6.60 \pm 0.26) \times 10^6$

The number of surviving cells was less than 70% in the other samples.

Most of the above samples (6 samples) included various concentrations of DMSO and fetal serum. After CEF cells were equilibrated with ethylene glycol and glycerin, one sample from each group was selected for further studies. The listed variants of the cell suspension were selected to study the CEF cell viability during storage at a temperature of minus 150 °C. Aliquots of cryopreservation mixtures containing cell suspensions, cryoprotectants and fetal serum in a volume of 4 cm^3 were placed in a foam thermal container and put for storage in a low-temperature freezer at minus 150 °C [16]. This method ensured cooling of the suspension at a rate of about 1 °C/min [12]. Thawing, cell count and seeding into culture flasks were carried out at intervals of one month. This step lasted 12 months. The test results of cell survival determination following cryopreservation at minus 150 °C are shown in Table 3.

The previously conducted studies showed that the highest survival rate of primary trypsinized CEF cells ranged from 53 to 71% [16]. After 12 months of storage, the maximum number of surviving cells (46%) was observed in case of a cell suspension containing serum in the amount of 20% and DMSO in the amount of 10% of the total volume. In a suspension containing serum and ethylene glycol at concentrations of 10% and 5%, respectively, the number of surviving cells decreased to 36%, however, this parameter was higher as compared to other samples containing various combinations of DMSO and serum. The number of live cells in the other suspensions containing DMSO and fetal serum decreased to 18–26% after 12 months of storage. The number of live cells in a suspension sample containing 10% serum and 5% glycerin decreased to 22% within 8 months. No live cells were detected in this sample after 9 months of storage. The smallest number of surviving cells was observed in the sample containing 10% serum and 15% DMSO. After one month of storage 24% of live cells were detected in that sample, the percentage of surviving cells decreased to 19% within the next 4 months of storage. No live cells were found in this mixture after 6 months of storage.

Subsequently, the proliferative properties of cryopreserved CEF cells were studied. After defrosting and counting, each sample cells were diluted with growth medium to a concentration of 600 thousand cells/cm^3 and seeded into culture flasks. The monolayer was daily observed under microscope, the duration of formation and the flask surface coating estimated in percentage were recorded [16].

Table 3
Survivability of CEF cells after storage at minus 150 °C using different cryprotectant samples ($n = 3$)

Storage period, months	10% serum and 5% DMSO	10% serum and 10% DMSO	10% serum and 15% DMSO	20% serum and 5% DMSO	20% serum and 10% DMSO	20% serum and 15% DMSO	10% serum and 5% glycerin	10% serum and 5% ethylene glycol
Before storage	10.0 ± 0.1	13.0 ± 0.1	9.0 ± 0.2	11.0 ± 0.3	8.0 ± 0.1	12.0 ± 0.3	8.0 ± 0.1	8.0 ± 0.1
1	3.3 ± 0.4 (33%)	4.9 ± 0.3 (37%)	**2.9 ± 0.2 (24%)**	4.2 ± 0.4 (38%)	**5.2 ± 0.3 (65%)**	5.7 ± 0.4 (47%)	**2.7 ± 0.2 (34%)**	**4.3 ± 0.2 (52%)**
2	2.9 ± 0.3 (29%)	4.3 ± 0.4 (33%)	2.9 ± 0.2 (24%)	4.0 ± 0.3 (36%)	4.9 ± 0.1 (61%)	4.9 ± 0.3 (41%)	2.5 ± 0.2 (31%)	3.5 ± 0.5 (43%)
3	2.9 ± 0.1 (29%)	4.0 ± 0.4 (31%)	2.1 ± 0.2 (23%)	4.0 ± 0.2 (36%)	4.6 ± 0.1 (57%)	4.8 ± 0.5 (40%)	2.5 ± 0.1 (31%)	3.3 ± 0.5 (41%)
4	2.8 ± 0.3 (28%)	3.0 ± 0.4 (23%)	2.1 ± 0.1 (23%)	4.0 ± 0.3 (36%)	4.3 ± 0.2 (54%)	4.2 ± 0.4 (35%)	2.4 ± 0.1 (30%)	3.3 ± 0.2 (41%)
5	2.6 ± 0.4 (26%)	3.0 ± 0.2 (23%)	**1.7 ± 0.3 (19%)**	4.0 ± 0.2 (36%)	4.3 ± 0.2 (54%)	4.1 ± 0.4 (34%)	2.1 ± 0.1 (26%)	3.2 ± 0.2 (40%)
6	2.5 ± 0.2 (25%)	3.0 ± 0.3 (23%)	0	4.0 ± 0.3 (36%)	4.2 ± 0.1 (52%)	4.0 ± 0.3 (33%)	2.1 ± 0.3 (26%)	3.1 ± 0.4 (39%)
7	2.3 ± 0.2 (23%)	3.0 ± 0.2 (23%)	0	4.0 ± 0.2 (36%)	4.0 ± 0.2 (50%)	4.0 ± 0.1 (33%)	2.0 ± 0.3 (25%)	3.0 ± 0.2 (37%)
8	2.2 ± 0.1 (22%)	3.0 ± 0.1 (23%)	0	3.8 ± 0.2 (34%)	4.0 ± 0.3 (50%)	3.9 ± 0.3 (32%)	**1.8 ± 0.3 (22%)**	3.0 ± 0.3 (37%)
9	2.0 ± 0.1 (20%)	3.0 ± 0.3 (23%)	0	3.5 ± 0.4 (32%)	3.9 ± 0.2 (49%)	3.9 ± 0.1 (32%)	0	3.0 ± 0.1 (37%)
10	1.8 ± 0.1 (18%)	3.0 ± 0.1 (23%)	0	3.5 ± 0.2 (32%)	3.9 ± 0.1 (49%)	3.7 ± 0.3 (31%)	0	3.0 ± 0.1 (37%)
11	1.8 ± 0.3 (18%)	2.9 ± 0.4 (22%)	0	3.2 ± 0.2 (29%)	3.8 ± 0.2 (47%)	3.6 ± 0.1 (30%)	0	2.9 ± 0.2 (36%)
12	1.8 ± 0.1 (18%)	2.3 ± 0.4 (18%)	0	2.9 ± 0.3 (26%)	**3.7 ± 0.1 (46%)**	3.2 ± 0.3 (26%)	0	**2.9 ± 0.1 (36%)**

Figure 1 demonstrates the data obtained at 1, 3, 6, 9, 12 months of storage. During testing of samples containing 10% serum and 15% DMSO, as well as 10% serum and 5% glycerin, the flask surface monolayer coating did not exceed 50%. The degree of flask surface coating with a CEF cell monolayer was 90–100% in other cases within 1–11 months of storage. At the end of 12 months of storage this value was 80%. The obtained values are optimal for the adequate cell culture monolayer.

The formation time of the monolayer in all CEF cell samples was 2–3 days, which is the optimal time for this process.

The final stage of this work was to study the susceptibility of CEF cells to viruses after exposure to low temperatures. CEF cells stored at minus 150 °C were thawed and seeded into culture flasks for monolayer formation. At the same time, a primary trypsinized CEF cell culture was used as control. The formed monolayer was infected with the reference strain of avian leukosis virus ALV-J ADOL-HCI-P7. Cell suspension samples containing 10% serum and 15% DMSO, as well as 10% serum and 5% glycerin, were not used at this stage of work due to weak proliferative properties. At the end of the incubation period, the concentration of virus-specific protein p27 was estimated using sandwich ELISA.

Figure 2 demonstrates the data obtained at 1, 3, 6, 9, 12 months of storage of cell samples as an example. The standard deviation ranged from 0.2 to 0.4. The virus accumulation was observed both in the primary trypsinized CEF cell culture and in the monolayer obtained from suspension of cryopreserved CEF cells. The concentration of the virus protein was 5–8 μg/cm^3. Throughout the study, a slight decrease in the concentration of virus protein was observed in a number of samples compared to the primary trypsinized CEF cell culture. The minimal discrepancy between this parameter and the value obtained for the primary trypsinized CEF cell culture was observed in a sample containing 10% serum and 5% ethylene glycol.

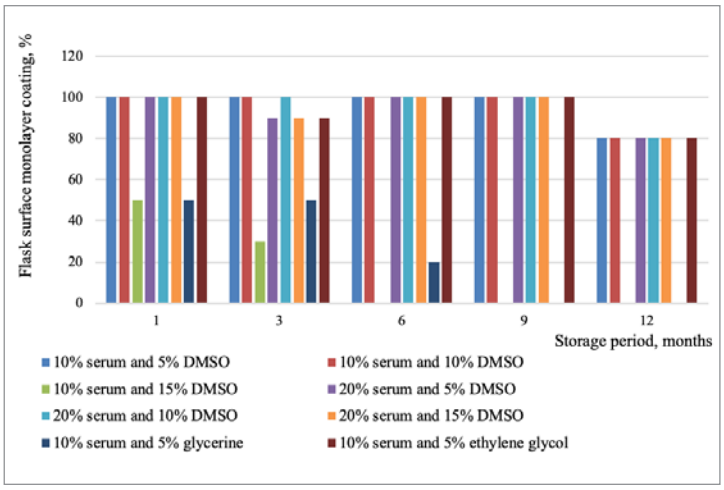

Fig. 1. Development (formation) of cell monolayer upon thawing

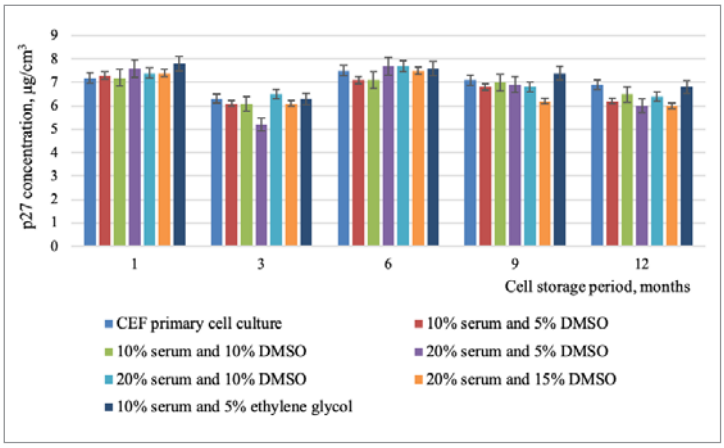

Fig. 2. Concentration of p27 viral protein after infection of CEF cell samples with ALV-J ADOL-HCI-P7 strain, μg/cm^3 (n = 3)

CONCLUSION

The study results revealed that the most optimal serum-cryoprotectant ratio for CEF cell suspension in deep freezing conditions is a combination of 20% fetal serum and 10% DMSO. The number of surviving cells in the cell suspension sample containing 10% fetal serum and 5% ethylene glycol was slightly lower, but it exceeded the same indicator in other samples. Glycerin added to the cell suspension did not provide sufficient cell protection from destructive effects of ultra-low temperatures. Proliferative cell properties and sensitivity to avian leukosis virus were preserved in the following cryoprotectant combinations: containing 10% bovine embryonic serum and 5, 10% DMSO, 20% bovine embryonic serum and 5, 10, 15% DMSO, as well as 10% bovine embryonic serum and 5% ethylene glycol. Cryoprotectant samples containing 10% serum and 15% DMSO, as well as 10% serum and 5% glycerin, showed the lowest protective property.

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Effectiveness and safety of therapeutics used for treatment of experimental or spontaneous *Mycoplasma* infections

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SUMMARY
Mycoplasmoses of cattle and small ruminants, pigs and poultry are widely spread and the infection process is frequently associated with other diseases. *Mycoplasma* spp. cause inflammatory respiratory diseases, diseases of joints and meninges, keratoconjunctivitis, mastitis and endometritis, abortion and stillbirths. Etiotropic therapy of mycoplasmal infections consists in prescribing antibiotics: enrofloxacin, difloxacin, oxytetracycline, chlortetracycline, doxycycline, tylosin, tilmicosin, tylvalosin, tiamulin, florfenicol, lincomycin, spectinomycin, tulathromycin. The results of studies described in different publications show high sensitivity of *Mycoplasma synoviae* and *Mycoplasma gallisepticum* to tetracyclines, tiamulin and tylvalosin. Isolates with increased resistance to tilmicosin are also resistant to tylosin and lincomycin. Treatment of respiratory infections in lambs, the main causative agents of which are *Mannheimia haemolytica* and *Mycoplasma*, has been successful with the use of fluoroquinolones, tilmicosin, tulathromycin, chlortetracycline, enrofloxacin, doxycycline, and oxytetracycline. Isolates of *Mycoplasma bovis* are largely sensitive to oxytetracycline, florfenicol and tulathromycin. Enrofloxacin has a less pronounced therapeutic effect. Tilmicosin and oxytetracycline are effective in the treatment of respiratory diseases of young cattle, associated with *Mycoplasma* spp. Tulathromycin and tilmicosin have a significant therapeutic effect in the treatment of pneumonia in weaned piglets experimentally infected with *Mycoplasma hyopneumoniae*. Multiple (course) use of enrofloxacin significantly increases the therapeutic effect. Tilmicosin is effective in the control of other bacterial infections of pigs (pasteurellosis, streptococcosis, hemophilic polyserositis, infectious atrophic rhinitis). The general prophylaxis of mycoplasmal infections is to comply with veterinary and sanitary standards and to implement quarantine measures in the infection outbreak.

Keywords: review, mycoplasmosis, treatment, poultry, small and large cattle, pigs, enrofloxacin, difloxacin, oxytetracycline, chlortetracycline, doxycycline, tylosin, tilmicosin, tylvalosin, tiamulin, florfenicol, lincomycin, spectinomycin, tulathromycin

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Эффективность и безопасность лекарственных препаратов при лечении экспериментальных и спонтанных микоплазменных инфекций

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РЕЗЮМЕ
Микоплазмозы крупного и мелкого рогатого скота, свиней и птиц имеют широкое распространение и чаще всего проявляются в ассоциативной форме течения инфекционного процесса. *Mycoplasma* spp. вызывают воспалительные заболевания органов дыхания, суставов и мозговых оболочек, керато-

конъюнктивиты, маститы и эндометриты, аборты и рождение мертвого нежизнеспособного приплода. Этиотропная терапия микоплазменных инфекций заключается в назначении антибиотиков: энрофлоксацина, дифлоксацина, окситетрациклина, хлортетрациклина, доксициклина, тилозина, тилмикозина, тилвалозина, тиамулина, флорфеникола, линкомицина, спектиномицина, тулатромицина. Результаты исследований, опубликованные в различных источниках, показывают высокую чувствительность *Mycoplasma synoviae* и *Mycoplasma gallisepticum* к тетрациклинам, тиамулину и тилвалозину. Изоляты с повышенной устойчивостью к тилмикозину также резистентны к тилозину и линкомицину. Лечение респираторных инфекций ягнят, основными возбудителями которых являются *Mannheimia haemolytica* и *Mycoplasma*, проходит успешно с применением фторхинолонов, тилмикозина, тулатромицина, хлортетрациклина, энрофлоксацина, доксициклина и окситетрациклина. Изоляты *Mycoplasma bovis* в значительной степени чувствительны к окситетрациклину, флорфениколу и тулатромицину, менее выраженный терапевтический эффект оказывает энрофлоксацин. При лечении респираторных заболеваний молодняка крупного рогатого скота, протекающих в ассоциации с *Mycoplasma* spp., эффективны тилмикозин и окситетрациклин. Значительное терапевтическое действие при лечении пневмонии у поросят-отъемышей, экспериментально инфицированных *Mycoplasma hyopneumoniae*, оказывает тулатромицин и тилмикозин, заметно повышает лечебный эффект многократное (курсовое) применение энрофлоксацина. Тилмикозин эффективен в борьбе и с другими бактериальными инфекциями свиней (пастереллезом, стрептококкозом, гемофилезным полисерозитом, инфекционным атрофическим ринитом). Общая профилактика микоплазменных инфекций заключается в соблюдении ветеринарно-санитарных норм и осуществлении карантинных мероприятий в очаге инфекции.

Ключевые слова: обзор, микоплазмоз, лечение, птицы, мелкий и крупный рогатый скот, свиньи, энрофлоксацин, дифлоксацин, окситетрациклин, хлортетрациклин, доксициклин, тилозин, тилмикозин, тилвалозин, тиамулин, флорфеникол, линкомицин, спектиномицин, тулатромицин

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Mycoplasmas cause many pathologies in humans, different animal and bird species: respiratory, autoimmune diseases, diseases of the reproductive organs and joints. It is also known that mycoplasmas infect the brain of sheep and goats, cattle, and birds. Evidence has been presented that some *Spiroplasma* species may play a role in the development of transmissible spongiform encephalopathies. Over the past few decades, mycoplasmas have been isolated from the brains of marine mammals dying in large numbers in the North Sea, although their role has been shown to be secondary to the primary viral disease [1]. Currently, mycoplasmas of aquatic animals are not well studied. A study by J. El-Jakee et al. shed light on the characterization of unique *Mycoplasma* isolates found in fish from various geographical areas throughout Egypt. *Mycoplasma* spp. were isolated using selective nutrient media and identified using morphochemical tests. Then 16S ribosomal RNA (rRNA) gene polymerase chain reaction (PCR) was performed for confirmation using molecular and genetic methods. The results showed that the incidence of *Mycoplasma* in *Cyprinus carpio*, *Oreochromis niloticus*, *Aulopiformes synodontidae* and *Clarias gariepinus* was 33.33; 16.36; 8.108 and 6.45%, respectively, while these microorganisms were not detected in *Mugil cephalus*. At the same time, mycoplasmas were found only in the gills and swim bladder of affected fish. Biochemically isolated mycoplasmas were grouped into two clusters: the first included 35 isolates, the second – 7 isolates. Mycoplasmas of the first cluster, in contrast to the representatives of the second cluster, actively reduced tetrazolium salts. A phylogenetic tree built on the basis of incomplete 16S rRNA gene sequences showed that both clusters are grouped into one branch and separated from other *Mycoplasma* spp., which indicates that both clusters belong to the same species. Interestingly, PCR with specific primers for the *M. mobile* and *M. monodon* species failed to identify all the *Mycoplasma* isolates recovered from fish. This result confirmed that the microorganisms of these two clusters belong to the unidentified *Mycoplasma* species, for which temporary names were introduced: *Mycoplasma* of the 1st cluster and *Mycoplasma* of the 2nd cluster. Pathogenicity tests of mycoplasmas of both clusters showed that after inoculation of Nile tilapia, all fish were susceptible to these *Mycoplasma* species [2].

Most often, mycoplasmas are the etiological factor of diseases in birds in Asian countries. So, when performing laboratory tests, C. J. Morrow et al. recovered 26 *M. synoviae* isolates and 11 *M. gallisepticum* isolates from 164 clinical specimens collected from China, India, Indonesia, Malaysia, Republic of Korea, Thailand and the Philippines. Most of the isolates were recovered from birds of industrial poultry farms. To increase the time of transportation to the laboratory and the safety of biological samples for tests, immediately after collection, the pathological material was purified by membrane filtration (pore size 0.45 μm). Minimal inhibitory concentrations (MICs) for enrofloxacin, difloxacin, oxytetracycline, chlortetracycline, doxycycline, tylosin, tilmicosin, tylvalosin, tiamulin, florfenicol, lincomycin, spectinomycin, and a combination of spectinomycin and lincomycin (1:2) were determined by broth microdilution. Some of the isolates showed reduced sensitivity to antimicrobials, not associated with antibiotic therapy. In general, the results obtained by the authors were similar to studies conducted worldwide on the study of antibiotic resistance of mycoplasmas. As a rule, high sensitivity of *M. synoviae* and *M. gallisepticum* to tetracyclines, tiamulin and tylvalosin was observed. Isolates of *M. synoviae* and *M. gallisepticum* with increased resistance to tilmicosin (MIC_{90} values ≥ 64 μg/mL) were also resistant to tylosin. All isolates with reduced sensitivity to lincomycin showed increased resistance to tilmicosin. It has been shown that the isolation of mycoplasmas and the

determination of MICs can also be carried out in farm laboratories, which will allow faster and more efficient use of antimicrobials or other methods of combating mycoplasmal infection in chickens (for example, live vaccines) and, therefore, more responsible use of antibiotics in terms of concept of "One Health" [3].

According to J. P. Yadav et al. data, mycoplasmosis is an economically significant disease in the poultry industry, which leads to huge losses, consisting of a decrease in weight gain, feed conversion efficiency, egg production, hatchability; increase in embryo mortality, carcass culling, costs for the prevention and treatment of broilers, laying hens and parent stock. The disease is caused by four main pathogenic mycoplasmas: *M. gallisepticum*, *M. synoviae*, *M. meleagridis* and *M. iowae*, which cause respiratory mycoplasmosis of chickens, infectious synovitis of chickens and turkeys, infectious sinusitis of turkeys, aerosacculitis of turkeys, infections of the genital organs of turkeys. Respiratory mycoplasmosis and infectious synovitis of birds caused by *M. gallisepticum* and *M. synoviae* are included in the list of notifiable diseases of the World Organization for Animal Health. Mycoplasmas are transmitted both horizontally and vertically. Prevention and control measures for avian mycoplasmosis mainly include biosecurity, treatment and vaccination. For vaccination of birds against infection caused by *M. gallisepticum* and *M. synoviae*, inactivated, live attenuated and/or recombinant (vector) live vaccines are used. The authors in their systematic review summarize various epidemiological studies conducted in 2010–2020 regarding mycoplasmal infections caused by *M. gallisepticum* and *M. synoviae* in poultry in various geographical locations in India and abroad, their economic impact, diagnosis, prevention and control [4].

One of the measures to combat *Mycoplasma* infection in productive animals is the use of antibacterial agents (tylosin, tiamulin, enrofloxacin, etc.), however, they cause the emergence of resistant strains of pathogens in herds and, if not prescribed correctly, can penetrate into people's food. *Mycoplasma* field strains are often detected in vaccinated herds, and the use of antimicrobials after vaccination against mycoplasmosis can affect the effectiveness of immunization with the vaccine strain, so this issue requires additional research.

In veterinary medicine, macrolide antibiotics, including tylosin and tilmicosin, are widely used for the prevention and treatment of mycoplasmosis. *In vitro* sensitivity testing of 50 strains of *M. gallisepticum* isolated in Israel between 1997 and 2010, conducted by a group of scientists led by I. Gerchman [5], showed that acquired resistance to tylosin, as well as to tilmicosin, is present in 50% of them. Moreover, 13 out of 18 *Mycoplasma* strains (72%) isolated from clinical samples since 2006 showed acquired resistance to enrofloxacin, tylosin and tilmicosin. Molecular typing of field isolates using targeted gene sequencing (GTS) revealed 13 *M. gallisepticum* molecular types (I–XIII). Type II was predominant until 2006, while type X, first discovered in 2008, is currently dominant. All ten type X strains were resistant to both fluoroquinolones and macrolides, indicating a selective pressure leading to the spread of clonal-type resistance. Resistant strains with other molecular types of resistance have also been found. At the same time, the molecular basis of *M. gallisepticum* resistance to macrolides was identified. The authors established a clear correlation between single point mutations at positions A2058G or A2059G in the 23S rRNA gene and acquired resistance of *M. gallisepticum* to macrolides. All isolates (MIC ≥ 0.63 μg/mL for tylosin and MIC ≥ 1.25 μg/mL for tilmicosin) have one of these mutations, indicating a significant role in reducing the sensitivity of *M. gallisepticum* to 16-mer macrolides. Similar results were obtained by other scientists [6, 7]. Infectious agalactia of goats and sheep caused by *M. agalactiae* is an infectious disease that requires rapid diagnosis in order to reduce the economic loss in milk production and the mortality of lambs [8]. To identify this etiological agent and take timely preventive measures, PCR is used. Using this method, J. F. De Almeida et al. examined 19 cultures stored for two years at –20 °C in modified Hayflick broth with 50% glycerol, seven of which were identified as *Mycoplasma* spp. and 12 were typed as *M. agalactiae* using an indirect immunoperoxidase assay [9].

The most common clinical signs of infectious agalactia in small ruminants are mastitis, conjunctivitis, and arthritis. Pregnant animals have abortions. The most susceptible to the disease are lactating animals, kids and lambs up to a month old. The main pathogens in sheep are *M. agalactiae*, in goats – *M. agalactiae*, *M. mycoides* subsp. *mycoides* and *M. capricolum* subsp. *capricolum*. In addition, *M. putrefaciens* can cause a similar clinical presentation, especially in goats. Infectious agalactia occurs on all five continents and frequently in the form of enzootic disease. Asymptomatic carriage of mycoplasmas is widespread, which is difficult to diagnose and control, while latent infection in the herd becomes chronic with a decrease in the level of immune protection. The release of the pathogen from the body into the external environment occurs mainly with milk and can last for a long time. The main route of the infection transmission is associated with the marketing of carrier animals and contact during cattle transhumance. The transmission of the pathogen within the herd occurs through direct contact with patients and *Mycoplasma* carriers through the mucous membranes, skin, digestive tract, and during milking [6, 10]. There are also reports of histopathological lesions in the brain of sheep experimentally infected with *M. agalactiae* through the mammary gland, which was the cause of non-suppurative encephalitis, as well as ataxia in young animals [1, 11].

Bacterial respiratory infections in lambs are quite common. Treatment should be aimed at controlling the clinical signs as well as limiting lung involvement in sick animals and requires immediate action, mainly with antimicrobial agents effective against the causative bacteria. In clinical practice, the correct identification of pathology in lambs is important for appropriate treatment. Fluoroquinolones, tilmicosin, tulathromycin, chlortetracycline, enrofloxacin, doxycycline, and oxytetracycline are effective against *Mannheimia haemolytica* and *Mycoplasma*, which are the main causative agents of respiratory infections in lambs [12–14]. The concomitant use of non-steroidal anti-inflammatory drugs is also recommended. All lambs with clinical signs should receive the full course of treatment. The potential value of metaphylactic treatment of clinically healthy lambs in affected herds should be assessed on a case-by-case basis. Disease management protocols should always include changes in herd management to eliminate factors contributing to the development of the disease [10].

According to D. Dacak et al., mycoplasmosis is considered a new disease in wild animal populations. A study published by the authors reports a case of mycoplasmosis in three

Procyon cancrivorus kept in captivity in the city of Asuncion (Paraguay). The diagnosis was established cytologically using Romanovsky-Giemsa-stained peripheral blood smears. Animals were treated with enrofloxacin (10 mg/kg), which led to a rapid recovery [15]. Information was also published on the detection by PCR of a new species of *Mycoplasma*, tentatively named *Mycoplasma pogonae*, in a bearded dragon (*Pogona vitticeps*), which died despite antimicrobial and supportive pneumonia therapy [16].

Mycoplasma spp. are unique microorganisms associated with several diseases, including mastitis, pneumonia, and arthritis in animals. One of the problems in determining the role of mycoplasmas in causing disease is their pathogenicity. *M. mycoides* subsp. *mycoide*s, *M. bovis*, *M. bovigenitalium* and *M. dispar* play a significant role in the development of mycoplasmosis in cattle. The study of vaginal swabs of cows in Brazil demonstrated that the detection rate of *M. bovigenitalium* was 9.29% [17], in Japan – 7.4% [7]. *Mycoplasma* can be isolated from both clinically healthy and diseased cattle. With natural infection in the field, mycoplasma pneumonia often occurs as a mixed infection. In addition, research observations and clinical experience have shown that the presence of *Mycoplasma* increases the severity of respiratory disease [14, 18–26]. *M. bovis* has been reported occasionally in the brains of calves and adult cattle with a range of histopathological lesions, including abscesses and fibrinous meningitis [1].

There are no pathognomonic signs of *Mycoplasma* infection. Clinical signs associated with respiratory infections include tachypnea, dyspnea, eye and nasal discharge, depression, decreased appetite, crooked posture, and fever. Clinical signs associated with joint infections include stiffness, lameness, difficulty standing up, swollen joints and tendon sheaths, decreased appetite, and weight loss.

Currently, among laboratory methods for diagnosing animal mycoplasmosis, serological blood testing for the presence of antibodies to mycoplasmas, PCR to detect mycoplasmal DNA in biological samples, as well as cultivation (bacteriological examination) followed by microscopy and study of the biochemical properties of recovered isolates are used. All three of these laboratory methods are often used simultaneously, as they complement each other. Bacteriological methods allow assessing the viability of mycoplasmas and at the same time have high sensitivity. *Mycoplasma* for cultivation requires special nutrient media and special conditions for growing in the laboratory. If the practicing veterinarian wants to confirm the diagnosis by microbiological isolation of mycoplasmas, when the samples are transferred to the laboratory the necessity of inoculation should be indicated. Upon receipt of a positive result for mycoplasmosis, the veterinarian should receive advice from the laboratory on the use of appropriate and effective drug treatments [27–29].

Therapy for mycoplasmosis, both experimental and in the field, is not always unambiguous and often does not bring results. Since mycoplasmas are resistant to a variety of drugs, the main focus should be on enhancing biological protection measures that minimize stress and exposure to animals and birds [30].

The impact of antimicrobial therapy and prevention strategies on respiratory disease in fattening cattle, as well as genetic relatedness and antimicrobial resistance of *M. bovis* isolates in western Canada, was studied by S. H. Hendrick et al. The feedlot calves (n = 3,784) were divided into groups. Some of the animals received oxytetracycline as a metaphylactic treatment, others, diagnosed with a respiratory disease caused by *M. bovis*, received florfenicol according to the scheme: once subcutaneously or twice intramuscularly with an interval of 48 hours, some animals did not use the antimicrobial drug. Calves from different treatment groups were pooled and observed for 100 days. Animals treated with oxytetracycline had a reduced risk of respiratory disease, an increased risk of arthritis, and no significant difference in average daily gain, disease recurrence, overall mortality, or mortality from respiratory infections. There were no significant differences between the treatment protocols. Swabs (n = 233) taken from the nasal mucosa before treatment (n = 122), during treatment (n = 77), smears from the lungs and joints at autopsy (n = 34) were collected from 61 animals that became ill or died from chronic disease (pneumonia and arthritis), as well as from 61 healthy calves. Next, bacteriological seeding was performed and *M. bovis* was cultivated. During the two years of the study, 51 isolates were recovered, which were tested for sensitivity to antimicrobials using special coated plates. The authors concluded that all isolates were significantly susceptible to the tested antimicrobials, except for tilmicosin, so it should not be used for the treatment of *M. bovis* mycoplasmosis without prior sensitivity testing [31].

In veterinary practice, the most popular is the use of drugs with prolonged action. Comparison of tilmicosin with long-acting oxytetracycline in the treatment of respiratory diseases in calves was studied by J. Musser et al. The purpose of the experiment was to compare the effect of a single parenteral injection of tilmicosin with the effect of a single dose of long-acting oxytetracycline as a treatment in the early stages of naturally acquired undifferentiated respiratory disease in young dairy calves. The experiment involved 40 calves of milk age from 5 farms, which were examined weekly until 3 months of age. When diagnosing respiratory disease, calves were assigned to one of two treatment groups. Samples of transtracheal swabs were collected to characterize pathogens. Then, within 3 days after treatment, the animals were examined and the severity of the disease course was assessed using a scoring system, and the growth rate was recorded. Given the body's response to initial treatment, disease relapse rate, and effect on growth rate, antibiotics were found to be equally effective. The manifestation of clinical signs of the disease was less pronounced ($P < 0.03$) in calves treated with tilmicosin on the 2nd and 3rd day after the start of treatment. During the tests of samples of transtracheal swabs, *Pasteurella multocida* was isolated from 25 out of 40 examined calves, *P. haemolytica* – from 4 animals, *Haemophilus somnus* – from 4, *Actinomyces pyogenes* – from 3 and *Aspergillus* spp. – from 2 calves. *Mycoplasma* was isolated in association with other bacterial isolates in 22 out of 40 calves examined. As a result of experiments, it was found that tilmicosin and oxytetracycline are effective in the treatment of respiratory diseases in young animals, even when *Mycoplasma* spp. are involved in the infectious process. Tilmicosin is more effective in eliminating the clinical signs of mycoplasmosis. Early treatment of dairy calves with respiratory diseases can reduce the negative impact on their growth and development [32].

The efficacy of tulathromycin versus enrofloxacin for the primary treatment of naturally acquired respiratory disease in fattening cattle was studied by E. J. Robb et al. Calves

with clinical signs of respiratory disease in two feedlots were randomly assigned to treatment with tulathromycin (2.5 mg/kg s.c.) or enrofloxacin (12.5 mg/kg s.c.). The use of tulathromycin resulted in a significantly higher ($P = 0.009$ and $P = 0.031$) therapeutic success (87.9 and 80.0%) than the administration of enrofloxacin (70.2 and 62.5%). Animals treated with tulathromycin received fewer follow-up treatments and also gained more weight compared to calves treated with enrofloxacin [33]. Other researchers also report the advantage of using certain antimicrobial drugs, as well as their combinations in other frequently recorded bacterial infections that occur both independently and in association with mycoplasmosis [34, 35].

An evaluation of the use of tulathromycin for the treatment of pneumonia after experimental intranasal infection of weaned piglets with *M. hyopneumoniae* was carried out by J. McKelvie et al. Five days after the inoculation of the pathogen, the animals were divided into groups: one received a single intramuscular injection of saline, the other received a single intramuscular injection of tulathromycin (2.5 mg/kg; day 0), the third received three intramuscular injections of enrofloxacin (5.0 mg/kg; days 0, 1, 2). The pigs were autopsied on the 12th or 13th day. Uninfected animals remained healthy without lung pathology. In pigs treated with tulathromycin, cough, mean lesion score, and proportional lung weight were significantly reduced, and weight gain was significantly greater compared to the control group ($P < 0.05$). When comparing the efficacy of enrofloxacin and tulathromycin, it was found that there were no significant differences in proportional lung weight or weight gain of piglets in the groups, but cough was worse and lung lesions were more severe in pigs treated with enrofloxacin ($P < 0.05$). The authors concluded that tulathromycin was effective in the treatment of pneumonia after experimental infection with *M. hyopneumoniae* [13]. Multiple (course) use of enrofloxacin significantly increases the therapeutic effect compared to three times, a number of researchers note [36–38].

A study of the efficacy and safety of tilmicosin phosphate in the treatment of experimental mycoplasmal infections in pigs was carried out by X. H. Zhang et al. Efficacy, recovery rate, mortality rate, severity of lung lesions were tested, and complete and biochemical blood tests as well as urinalysis were performed. The results showed that the administration of 10% soluble tilmicosin phosphate powder at doses of 100, 80 and 60 mg/L had a distinct therapeutic effect in pneumonia of pigs of mycoplasmal etiology (lesions in the lungs decreased significantly). In addition to the pronounced antibacterial action, the drug contributed to an increase in the weight of sick pigs. The authors noted that treatment of infected pigs with tilmicosin phosphate at a dose of 60–100 mg/L did not affect the results of blood and urine tests, and therefore it is safe for sick pigs [39].

The effectiveness of a macrolide antibiotic in reducing the number of respiratory pathogens in piglets weaned from sows 12 and 21 days after farrowing was evaluated by L. K. Clark et al. The aim of their studies was to determine the therapeutic effect of a feed antibiotic (tilmicosin) on pigs infected with *M. hyopneumoniae*, as well as the effect of the drug on other respiratory pathogens. The experiment used fifty pigs, divided into five experimental groups. Piglets of three groups were weaned from sows at 12 days of age: one was infected with *M. hyopneumoniae* and treated with tilmicosin; the second was infected, but the drug was not administered; the third group was intact. Piglets of two more groups were weaned from the sow at the age of 21 days, they were not subjected to infection with *M. hyopneumoniae*, while tilmicosin was used in one group, and not used in the other. Some pigs in all treatment groups developed clinical signs similar to those of *Haemophilus parasuis* disease and were injected with penicillin for 3 consecutive days. The development of respiratory disease was assessed by the presence of cough and lung lesions at autopsy. The biological material was tested for *M. hyopneumoniae*, *Actinobacillus pleuropneumoniae*, *H. parasuis*, *Pasteurella multocida*, *Streptococcus suis*, and *Bordetella bronchiseptica*. In addition, the sera of all pigs were tested for antibodies to *M. hyopneumoniae* and *A. pleuropneumoniae*. Body weight was also measured and growth was calculated in the period from 12 to 56 days. The authors found that tilmicosin did not affect the growth rate of pigs of different groups and reduced cough ($P < 0.01$), although the degree of lung damage was slightly ($P > 0.05$) different from animals that did not receive the drug. *A. pleuropneumoniae*, *B. bronchiseptica* and *P. multocida* were not isolated from any of the pigs.

Four of the seven piglets from which *S. suis* was isolated were from the control group, while the pathogen was not detected in animals treated with tilmicosin. *H. parasuis* was isolated at autopsy from 19 of 20 pigs uninfected with *M. hyopneumoniae* and 7 of 30 early weaning pigs. Pigs in all five groups were seropositive for *A. pleuropneumoniae* at 12 days of age, but titers decreased over the course of the experiment. Two out of ten control pigs seroconverted to *M. hyopneumoniae*. It was concluded that tilmicosin reduced the lesions caused by the disease mycoplasma pneumonia, delayed the onset of cough and, probably, thus prevented the development of lung pathology, reduced colonization of *S. suis* and seroconversion of *M. hyopneumoniae* [36].

The benefits of using tilmicosin in other frequently reported bacterial infections (pasteurellosis, streptococcosis, hemophilic polyserositis, infectious atrophic rhinitis) are also reported by other researchers [39–41].

CONCLUSION

Mycoplasmas are able to cause deep pathological processes in the human body, animals of various species and birds. They cause inflammatory diseases of the respiratory system, genitourinary system, joints, meninges. Most often, mycoplasma respiratory infection occurs in the form of pneumonia and can complicate the course of any viral respiratory infection.

Mycoplasmosis is an economically important disease in the poultry industry that causes huge losses. The results of studies published in various sources show that, as a rule, high sensitivity of *M. synoviae* and *M. gallisepticum* is observed to tetracyclines, tiamulin and tylvalosin. Isolates with increased resistance to tilmicosin (MIC_{90} values ≥ 64 μg/mL) are also resistant to tylosin and lincomycin. Israeli scientists, when testing the *in vitro* sensitivity of 50 strains of *M. gallisepticum* to antimicrobial drugs, found that acquired resistance to tylosin, as well as to tilmicosin, is present in 50% of them. The authors found a clear correlation between single point mutations at positions A2058G or A2059G in the 23S rRNA gene and acquired resistance of *M. gallisepticum* to macrolides.

The fluoroquinolones, tilmicosin, tulathromycin, chlortetracycline, enrofloxacin, doxycycline and

oxytetracycline are effective against *Mannheimia haemolytica* and *Mycoplasma*, which are the main causative agents of respiratory infections in lambs.

An analysis of the available literature showed that *M. bovis* isolates are largely sensitive to oxytetracycline, florfenicol, and tulathromycin; enrofloxacin has a less pronounced therapeutic effect. As a result of experiments, it was found that tilmicosin and oxytetracycline are effective in the treatment of respiratory diseases in young animals, even when *Mycoplasma* spp. are involved in the infectious process. Tilmicosin is more effective in eliminating the clinical signs of mycoplasmosis, but should not be prescribed without prior pathogen sensitivity testing.

When comparing the effectiveness of drugs for the treatment of pneumonia after experimental intranasal infection of weaned piglets with *M. hyopneumoniae*, a significant therapeutic effect of tulathromycin was established, and the therapeutic effect was significantly increased by multiple (course) use of enrofloxacin. The use of broad-spectrum drugs, which include tilmicosin, is also promising in the treatment of mycoplasmal infections in pigs. Tilmicosin was effective in combating other commonly reported bacterial infections in pigs (pasteurellosis, streptococcosis, hemophilic polyserositis, infectious atrophic rhinitis).

Thus, the effectiveness of the treatment of mycoplasmosis in birds, cattle and small cattle, pigs depends both on the drugs used and on the etiological agents, while infections associated with mycoplasmosis play a significant role.

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Biological properties of coronaviruses of farm, domestic animals and birds: comparative characterization of pathology they induce (review)

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SUMMARY

Coronavirus induced diseases can cause significant damage to agriculture that is associated with high (up to 100%) lethality in young animals. Members of the family *Coronaviridae* are characterized by the fact that they infect a wide range of animals and birds with expressed species-limited pathogenicity. One more coronavirus specificity involves their ability to simultaneously affect more than one organ. The disease severity is also strongly correlated with the age of the susceptible animal and degree of pathology. Thus, the coronavirus induced diseases are most often acute in newborn and young animals, while such diseases often develop into chronic and latent forms in adult animals. The general property of all coronavirus-induced diseases involves acute impairement of capillary circulation in the affected organ thus leading to the development of further pathology. The proposed review demonstrates brief overview of the history of discovery and examination of the viruses of *Coronaviridae* family and describes the coronavirus taxonomy. The paper reviews the virus structure, physico-chemical and biological properties; it describes specific features of their cultivation *in vitro*, some biochemical aspects of the virus replication and analyses the process of their propagation in the sensitive cells. Some data on the virus antigen structure and immunogenicity, on the presence of related antigens in the coronaviruses infecting humans, animals and birds are demonstrated as well. The paper provides data on the significant role the coronaviruses play in the pathology of farm animals and stresses their economic relevance, in particular for the commercial pig and poultry production.

Keywords: review, coronaviruses, mammals, avian, immunity, clinical picture

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Биологические особенности коронавирусов сельскохозяйственных, домашних животных и птиц: сравнительная оценка характера вызываемых ими патологических процессов (обзор)

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РЕЗЮМЕ

Болезни, вызываемые коронавирусами, могут наносить значительный ущерб сельскому хозяйству, обусловленный высокой (доходящей до 100%) летальностью среди молодняка. Представители семейства *Coronaviridae* характеризуются тем, что поражают широкий спектр животных и птиц, при этом отмечается выраженное видовое ограничение патогенности. Еще одной особенностью коронавирусов является способность поражать не один, а сразу несколько органов. Также существует четкая зависимость тяжести течения болезни от возраста восприимчивого животного и интенсивности патологических процессов. Так, заболевания, вызываемые данными вирусами, чаще всего имеют острое течение у новорожденных животных и молодняка, у взрослых особей они нередко переходят в хроническую и латентную формы. Общее свойство всех болезней, индуцированных коронавирусами, – острое нарушение капиллярного кровообращения в пораженном органе, становящееся основой развития дальнейшего патологического процесса. В предлагаемой обзорной статье представлена краткая информация об истории открытия и изучения вирусов семейства *Coronaviridae*, приведена таксономия коронавирусов. В работе рассматривается строение, физико-химические и биологические свойства данных вирусов, изложены особенности их культивирования *in vitro*, некоторые биохимические аспекты репликации, анализируется процесс размножения в восприимчивых клетках. Кроме того, обобщены некоторые данные об антигенной структуре и иммуногенности, о наличии родственных антигенов у коронавирусов, поражающих человека, животных и птиц. В статье приводятся данные о значительной роли коронавирусов в патологии сельскохозяйственных животных и подчеркивается их экономическое значение, особенно в условиях промышленного свиноводства и птицеводства.

Ключевые слова: обзор, коронавирусы, млекопитающие, птицы, иммунитет, клиническая картина

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INTRODUCTION

While considering the background of the issue one should mention that coronaviruses infecting animals and mostly birds were detected and studied before the human coronaviruses. Some of them were actively investigated and used as a model system in the studies of the molecular biology of the whole group of these viruses [1].

The first representative of the coronavirus family was isolated during the studies of avian infectious bronchitis etiology, and it was Infectious bronchitis virus (IBV). The viral nature of the highly contagious disease, often lethal for young chickens, was substantiated by the American researchers [2, 3]. Other researchers later detected the viral particles by means of negative-contrast electron microscopy of the IBV suspension [4]. The viral particles were mostly of round or oval shape and possessed thick membrane with club-shaped protrusions – spikes (Fig. 1).

In later years, the new family was persistently supplemented with the infectious agents isolated from humans and animals [5]. Starting from 1965, publications appeared that reported on the virus isolation from diseased people demonstrating acute respiratory signs [6]. One of such viral agents isolated from the diseased teenager and subjected to three passages in volunteers was identified as B814. This strain and strains 229E, OC38, OC43, isolated by the American researchers D. Hamre and J. J. Procknow [7] in human embryonic kidney cell line turned out to be similar in structure. Their further examination demonstrated common morphology of the isolates and IBV. Therefore, that enabled their incorporation in the same taxonomic group.

Comparative studies carried out by the group of researchers demonstrated such integration. It was additionally confirmed that murine hepatitis virus can be also included in this group as it has similar shape and structure [8].

Despite different natural hosts and diseases caused, they share not only structure but also a number of biological properties. Five key identification criteria were selected:

– average size of the virion is 80–160 nm;
– single-stranded RNA;
– membrane;
– virions are shaped as rounded bodies with typical crown of club-shaped protrusions;
– virus replication in cytoplasm with budding into the cytoplasmic vacuoles.

In 1968, the group of infectious agents having similar structure were proposed to be named coronaviruses thus emphasizing their specific shape. In 1976, the International Committee on Taxonomy of Viruses (ICTV) assigned the coronaviruses the status of the family. Hereafter, the family was supplemented with other viruses isolated from animals and birds as well as agents detected as a result of studies of contamination of cell cultures of different origin.

GENERAL DESCRIPTION OF CORONAVIRUSES

Coronaviruses (CoV; family *Coronaviridae*) belong to order *Nidovirales*, which includes large enveloped RNA-viruses. According to D. K. Lvov et al., this order comprises three families, one of which (*Coronaviridae*), in its

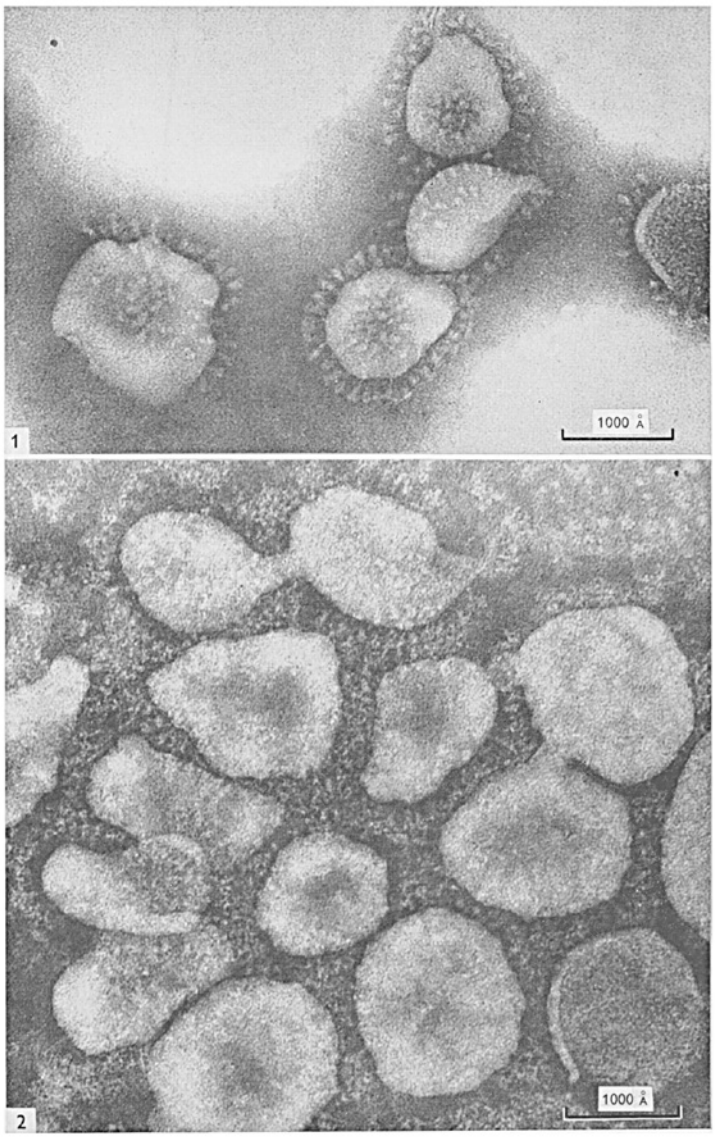

Fig. 1. General view of the infectious bronchitis virus virion. Negative staining [4]

turn, consists of two subfamilies and eight genera [9]. The ICTV proposed to use Greek alphabet letters (alpha, beta, gamma and delta) for designation of four genera of the coronavirus family [10]. As mentioned above, the viruses belonging to the family *Coronaviridae* have common biological properties and they are similar in their morphology. The coronavirus virion has a spherical shape (120–160 nm) and typical protrusions – peplomers (15–20 nm) forming serrated framing. Their shape and distance between the club-shaped protrusions serve as the differential structural properties, which allow to distinguish between coronaviruses and ortho- and paramyxoviruses by negative staining.

Coronavirus nucleocapsid is a long flexible helix containing the genomic positive-sense RNA (molecular weight 5.5×10^6) and a large number of molecules of phosphorylated nucleocapsid protein N (50–60 K). The virus envelope consists of lipid biolayer formed of the intracellular membrane of the host cells and two viral glycoproteins: E1 (20–30 K) and E2 (180–200 K); peplomers are formed of E2 molecules. The matrix glycoprotein E1 passes through the lipid biolayer and interacts with the nucleocapsid inside the viral particle [11] (Fig. 2).

PHYSICAL AND CHEMICAL PROPERTIES OF THE CORONAVIRUSES

Coronaviruses are infectious agents with moderate persistence in the environment. All members of the family have high tolerance for the vacuum freeze-drying, and being freeze-dried they can survive for several years at 4 °C. The coronaviruses can be UV-inactivated. The period necessary for the complete loss of infectivity depends on the distance from the UV-exposure source. Longer exposition (100–120 min) results in complete destruction of the virion of the porcine hemagglutinating encephalomyelitis virus (PHEV), loss of its infectivity and hemagglutination activity [12]. Sun rays also destruct the coronaviruses, however this procedure is rather slow, at least 3 hours at 37–38 °C are necessary for the complete inactivation of the virus [13].

Data on the hydrogen ion effect on the coronavirus stability are diverse. The majority of the researchers hold to an opinion that pH range from 7.0 to 7.5. is optimal for all members of the family. All coronavirus species are destructed by the fat solvents: when treated with Tween, ether, chloroform the viruses are completely inactivated [14].

Ionic and non-ionic detergents (Triton X-100, sodium dodechyl sulphate, deoxycolate, Nonidet P-40) destruct coronaviruses [15]. Members of this coronavirus family are relatively resistant to proteases (trypsin, pepsin) and amylases, but they are destructed by phospholipase C.

The coronaviruses are inactivated by the following disinfecting agents: 1% solutions of phenol, cresol and formalin, 70% ethanol, carbolic soap solution and 10% soda solution, upon 3–10 min exposure [16].

The coronavirus survival in the environment was modeled on IBV by T. T. Satylganov, and the test demonstrated the coronaviruses maintained infectivity for 8–10 hours in aerosols, much longer in drinking water (up to 9 days) and up to 12 days on hard and soft objects (down, feathers, eggshells, wood) [17].

Outdoors the virus inactivates within 4–11 days in spring at 0–18 °C and relative humidity 49–84%; in 3–9 days in summer at 10–23 °C and humidity 68–90%; in winter the virus maintains activity for 33–44 days. Six hours were required for effective disinfection in case of treatment of infected surfaces with bleach solution containing 3% of active chlorine; it took three hours in case of treatment with 0.5% formaldehyde and 3% tetrachloride, while sodium hypochlorite solution with 1.5% active chlorine resulted in disinfection of the surfaces in one hour. Exposure time increased with the use of aerosols. Thus, safe IBV inactivation required 12-hour exposure of the disinfected surface to the sodium hypochlorite aerosol (5% active chlorine) [17].

CORONAVIRUS REPRODUCTION FEATURES

Biochemical aspects of coronavirus replication were first studied by W. B. Becker et al. while examining IBV infected chick embryo chorioallontoic membrane (CAM) cells [18]. The virus was found to bud into the cisterns of cytoplasmic reticulum and cytoplasmic vesicles but not

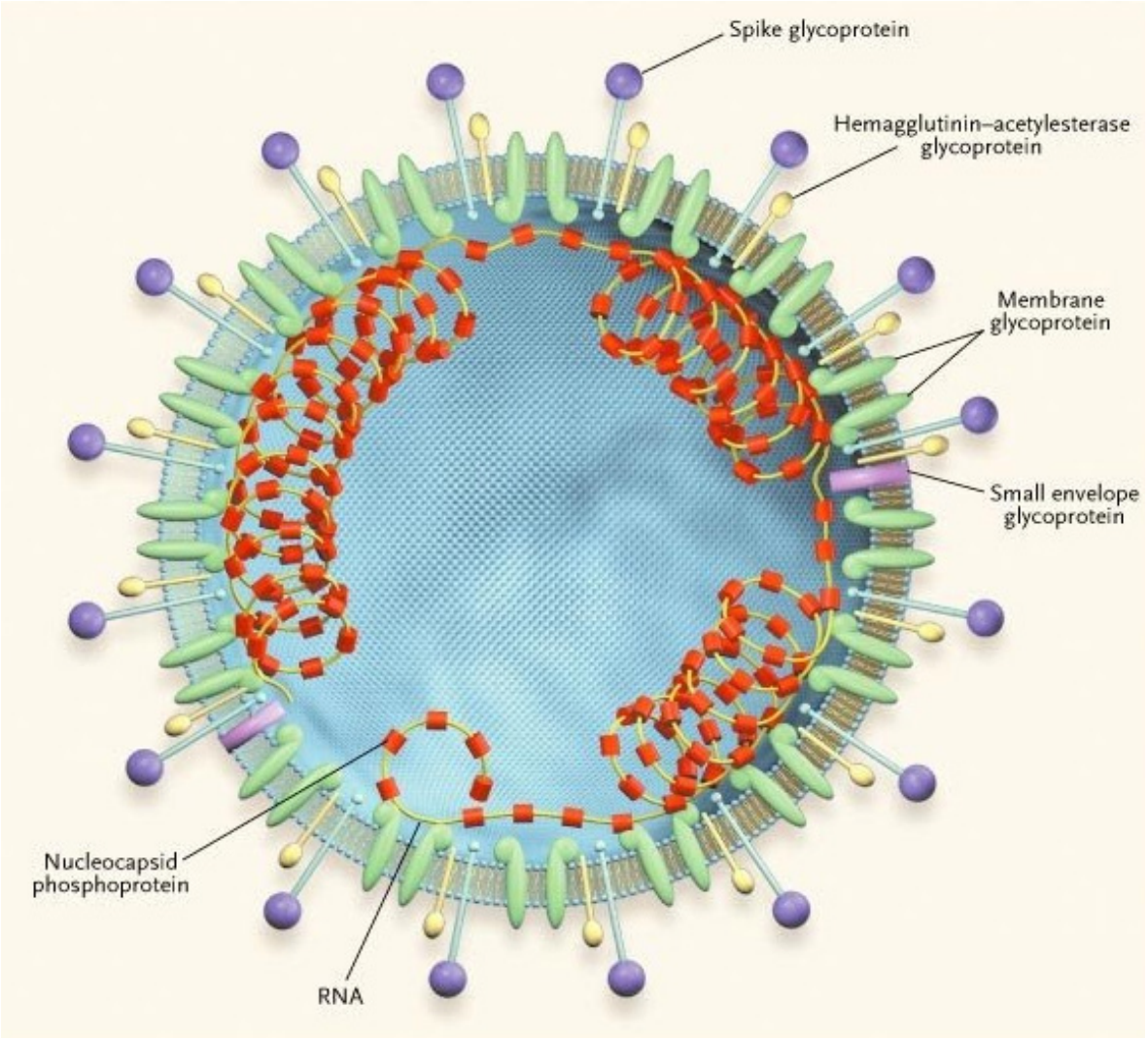

Fig. 2. Coronavirus structure [11]

through the plasmolemma. Before budding, an expressed thickened crescent-shaped layer of the protein appears on the membrane [19, 20]. Results of further studies performed by different researchers demonstrated that the coronaviruses attach to the target cell receptors with their E-2 peplomer termini [21–23]. The virus penetrates into the cell by fusion of the viral envelope with the plasmolemma or by endocytosis [1].

For all positive RNA-containing viruses the first stage of the virus cycle after the penetration into the cell involves attachment of the genomic RNA to ribosomes that results in the synthesis of the viral RNA-dependent RNA-polymerase [24].

Nucleocapsid N protein and some non-structural proteins are obviously synthesized on the polysomes in the cytoplasmic matri [25, 26]. Synthesis of E1 and E2 glycoproteins occurs on the polysomes attached to the rough endoplasmic reticulum (RER). Coronavirus spiral nucleocapsid is formed in the cytoplasm of the infected cells due to interactions between the newly synthesized RNA with N protein molecules. Nucleocapsid possesses flexible and rather loose structure, and it is sensitive to RNAse, its density amounts to 1.24–1.29 g/cm^3 [27, 28].

Coronavirus virions are formed by budding from RER membranes and/or Golgi apparatus [18] (Fig. 3).

On RER or Golgi apparatus membranes the host cellular proteins are eliminated from the budding virions and replaced by the viral glycoproteins; nucleocapsid and RER or Golgi apparatus membranes interact through E1 glycoprotein cytoplasmic domain. Coronavirus budding occurs only on the intracellular membranes, where E1 molecules are localized [29–32]. Large amount of E2 can be accumulated on the plasmatic membrane, but virion budding never occurs here, probably due to the lack of free E1. Nucleocapsid-containing budding virions "squeeze" into the RER and Golgi apparatus cavity and move to smooth bulbs (vesicles) and migrate to the edge of the cell, where they fuse with the plasmatic membrane resulting in the release of a large amount of virions in the extracellular space [14, 33, 34].

The virions often release from the cell only after its death, the coronaviruses however can release from the non-destructed cells, evidently, by means of cell secretion mechanism [31, 32, 35, 36]. The ability of the coronaviruses to release from the unlysed cell is the key factor for potential moderate (non-cytopathic) infection. Great number of

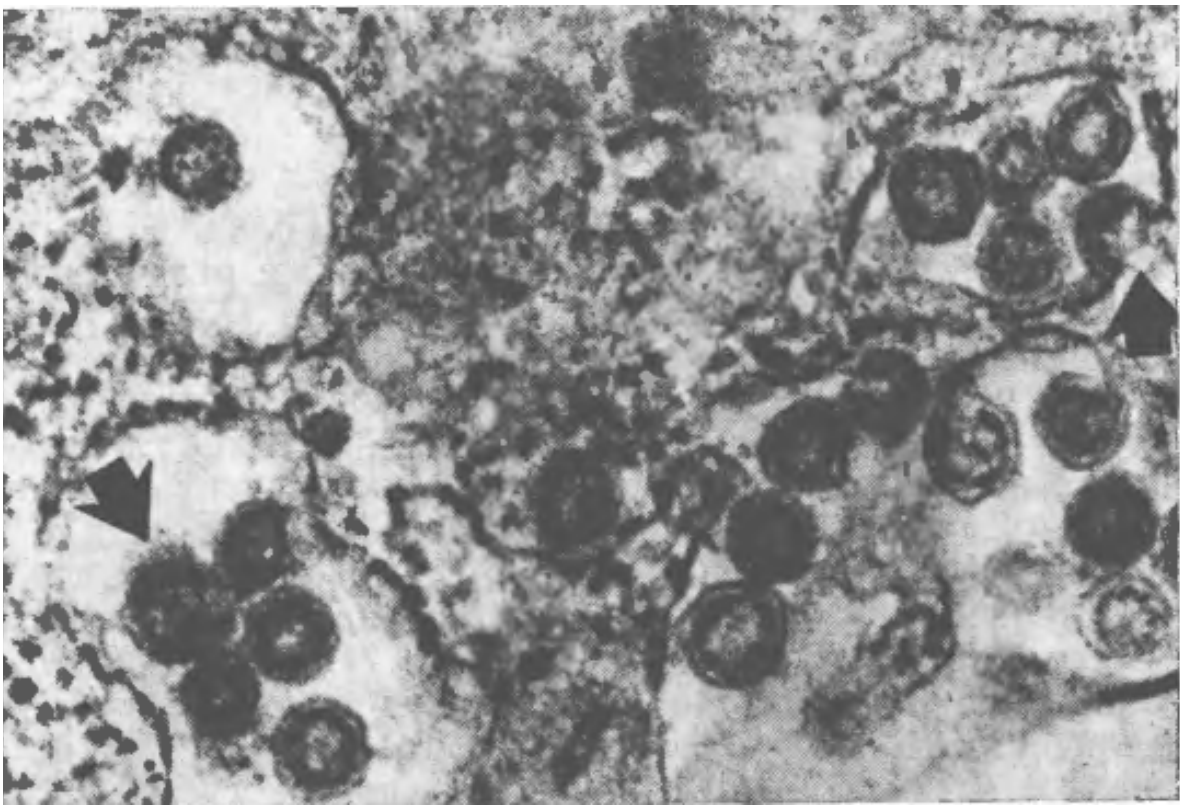

Fig. 3. A cell infected with the human respiratory coronavirus HCoV-229E. Budding of the coronavirus spherical virions (arrows) is observed on the membranes of RER and smooth vesicles. Inside the virions, electron-dense nucleocapsids are visible. Before leaving the cell due to exocytosis or cell lysis, the virions enter the RER cavity or pass through the Golgi apparatus (magnification 60,000×) [21]

virions were observed on the plasmatic membrane of the infected cells and they did not bud from it but most likely adsorbed to it after the release from the infected cells [37].

IMMUNOLOGICAL ASPECTS OF CORONAVIRUS INFECTION

The coronaviruses as well as other enveloped viruses have a complex antigenic composition. Studies of humans and animals recovered from the natural coronavirus infection or artificially infected with the coronaviruses attest to the fact that the antigens are capable of inducing virus neutralizing, complement-fixing, precipitating, lytic and antihemagglutinating antibodies. Immune response develops quite rapidly: relatively high antibody titers can be reported in blood by day 10–15 post infection or immunization. Antibody action mechanism was studied by direct electronic microscopy. Thus it was demonstrated that sera of convalescent or immunized birds not only had virus-neutralizing properties but they were also capable of inducing clearly microscopically visible agglutination of the coronavirus virions [4]. Moreover, different effects of the homologous and heterologous sera were established thus being indicative of the uneven structure of the coronavirus membranes and presence of definite loci, where the most active antigens are accumulated. Virus-encoded specific whole antigens are localized on the distal termini of the spikes. This data obtained from IBV examination were supported in other avian coronavirus model – turkey enteritis virus [38].

General knowledge of the antigenic properties of different coronavirus species are based on comparative studies of the relationship between various strains performed using neutralization test, complement fixation test and less frequently by hemagglutination inhibition test as well as by observation of cross-resistance effect *in vivo* [4, 14].

According to some reports, the coronaviruses causing porcine encephalomyelitis, transmissible gastroenteritis of swine (TGE) and porcine epidemic diarrhea (PED) are antigenically independent species, but according to other reports they contain common antigens [39–48]. Comparative studies of PHEV strains isolated in different countries including USA, England and Japan, demonstrated their antigenic structure similarity, and they were ranged in the same serotype [39, 40]. No antigenic variability was determined for TGE virus: strains isolated from animals in different countries were serologically identical [41]. In 1986, TGE-like porcine respiratory coronavirus (PRCV) was isolated and identified, which was antigenically related to TGE virus and induced TGEV-neutralizing antibodies. The key difference of the new coronavirus included its extraordinary pneumotropism with lack of enteropathogenicity [42, 47]. Both viruses are antigenically similar and have neutralizing epitopes in the basic virion proteins (N, S and M) [47].

Among the mammalian coronaviruses TGEV demonstrates relatedness to canine enteric coronavirus, feline infectious peritonitis virus and strain 229E-associated respiratory coronavirus in humans [43, 44]. The antibodies induced in the blood of dogs following the coronavirus infection are capable of TGEV neutralization [45].

Knowledge of antigenic and genomic homology of *Coronaviridae* family members are based on comparative studies involving different up-to-date methods. As a result, type-specific (within the same species), group-specific (within a group of antigenically related viruses: TGEV and PRCV) and interspecies (canine, feline, porcine coronaviruses) antigenic relatedness was determined.

Antigenic relatedness of human and animal coronaviruses are of particular interest. Human coronaviruses were determined to be serologically related to bovine and murine coronaviruses as well as to PHEV [1].

Coronaviruses of rodents (murine hepatitis virus and rat coronavirus) are antigenically related to each other and to the human coronavirus [49].

Enteropathogenic coronaviruses of turkeys and bovines also demonstrate antigenic relatedness. Bilateral antigenic relatedness was determined for turkey coronavirus and chicken and murine coronaviruses.

Genetic recombination was reported to be the most frequent between the genomes of different but related coronaviruses. This can be an important mechanism for the natural occurrence of genetically-modified viruses [47].

ROLE OF CORONAVIRUSES IN ANIMAL AND AVIAN PATHOLOGY

The major coronavirus-induced disease in pigs is *transmissible gastroenteritis* manifested with vomiting, profuse diarrhea, systemic dehydration and high lethality, particularly in piglets during the first 10 days of life. During primary outbreaks, the morbidity and mortality of newborn piglets can reach 100%, in such case the farm remains nearly without litter [33, 44].

TGE pathogenesis is featured by its ability to reproduce in the respiratory tract of the pigs as well, i.e. in the epithelial cells of the nasal and pulmonary mucosa [50, 51]. *In vitro* experiments demonstrated that TGEV replicates in porcine macrophage alveolar cell culture. This supports the fact that the virus infects not only intestines [44, 52–54].

Fattening pigs are considered to be the most probable TGEV reservoir in inter-epidemic period, who maintain the disease in enzootic and asymptomatic forms [44]. Reservoirs of the infection agents can be the herds, where weekly farrowing system is practiced [55].

Another porcine coronavirus with hemagglutinating activity induces severe disease, and it is currently known as *porcine encephalomyelitis* virus [34, 49, 56]. The outbreak of the disease with encephalomyelitis clinical manifestation was first described in 4–7-day-old pigs in Ontario (Canada) by A. S. Greig et al. [57].

Porcine hemagglutinating encephalomyelitis virus was isolated from the diseased newborn piglets in Poland in 1971 as well as from nasal conchae of pigs demonstrating atrophic catarrh in the USA in 1972. This coronavirus infection is specified by high contagiousness (nearly 100%), and it is manifested with vomiting, anorexia, constipation and progressive cachexia in animals. The disease mostly affects pigs of over 2 weeks of age [57]. According to C. K. Roe and T. J. Alexander, mortality in pigs of such age reaches 100% [58], and results of the studies performed by other researchers indicate that this parameter somewhat varies [59]. The disease incubation period generally lasts for 5–6 days. Sometimes, vomiting and anorexia are the key symptoms during the first 2–3 days, however, later on signs of severe CNS lesions come to the fore.

The virus replicates in the porcine thyroid cell culture, porcine embryo lung cells, in the continuous neonatal porcine testis cell culture and in porcine kidney cell line PK-15. During reproduction in the porcine kidney cell culture giant cells are formed [60].

Coronavirus enteritis in calves. In 1972 the American researcher C. A. Mebus et al. for the first time established possible coronavirus origin of dyspepsia in newborn calves [61, 62]. Primary etiologic role of the agent was proven during the experimental infection of calves with coronavirus in the natural environment as well as during the experimental infection of newborn, colostrum deprived calves and gnotobionts [63].

Whereas the most typical clinical sign of coronavirus enteritis in calves involves diarrhea, the virus infects not only the intestines but also the respiratory tract of the animals. The agent mostly replicates in distal part of the small intestine and colon, epithelial cells of nasal, tracheal and lung mucosa. Bovine coronaviruses can induce latent infections. Clinically healthy animal can be chronic virus carrier and it can shed the virus with the feces for seven months [64].

Mebus C. A. et al. described pathological and anatomical lesions in gnotobiotic calves infected with coronavirus enteritis agent [61]. The disease progression was supported by immunofluorescence tests and electronic microscopy of ultra-thin sections of intestinal walls of calve demonstrating acute diarrhea signs, which were emergently slaughtered in different periods post infection including those slaughtered at the peak of clinical manifestation. Specific fluorescence was reported in the epithelial cells of the small intestinal villi already in 4 hours post infection and reached its maximum by 44 hours post infection. Electronic microscopy of small intestine mucosal cells demonstrated virus particles in cytoplasmic vacuoles, large cisterns and pericellular space in the reticuloendothelial cells of the mesenteric lymph nodes [65, 66].

Infectious bronchitis (IB) virus is the best studied representative of avian coronaviruses. The basic clinical signs of the disease include air sac inflammation, rhinitis, mucous sputum producing cough, sneezing, conjunctivitis, dyspnea, depression. The disease incubation period is short: it averages between 18 and 36 hours. Birds of different ages have different disease: mortality of 1–5-week-old chicks reaches 25–40%. The disease is highly contagious and already on the first days post outbreak onset, 70–90% of chicks develop expressed clinical signs. In adult layers the disease is specified by less expressed respiratory signs and general low mortality. The birds demonstrate apathy, loss of appetite, sneezing, nasal discharge and egg drop [19, 67, 68]. It is egg drop that is considered to be the most harmful effect of the coronavirus infection.

Coronavirus enteritis (blue comb) of turkeys. The disease was first reported in the USA in 1951. In 1953 the virus was isolated, which was ranged in coronavirus family in 1974.

In all cases the disease was reported as highly contagious and it affected 90–100% of chicks and adult birds [70]. The outbreaks as a rule occur spontaneously and already in 3–5 days all susceptible population becomes infected. The incubation period lasts for 48–72 hours. The disease starts with the temperature rise and the turkeys refuse from feed and water; gastrointestinal disorder (diarrhea), weakness, cyanosis of the comb and body weight loss are reported [71].

Feline infectious peritonitis is a highly contagious disease, which was first described by American researchers L. G. Wolfe and R. A. Griesemer in 1966 [72]. Its most typical signs include ascites, anorexia, body weight loss, temperature fluctuations accompanied with depression. Some animals demonstrate vomiting, diarrhea, anemia and jaundice. The disease generally develops slowly and lasts for 3–4 months. The mortality of the diseased cats is pretty high. Using electron microscopy of tissues of the euthanized cats B. C. Zook et al. demonstrated virus particles morphologically similar to the coronaviruses in the cells of the liver, spleen and intestines [73]. J. M. Ward proved etiological relation between these agents and feline infectious peritonitis virus [74].

Etiological agent of *canine coronavirus enteritis* was for the first time isolated in USA in 1971. The virus is well replicated in the canine kidney cell culture. Experimental infection of puppies resulted in diarrhea due to the destruction of mature cells of the villi of the small intestines. Canine coronavirus is antigenically related to the porcine transmissible gastroenteritis virus, porcine respiratory coronavirus and feline infectious peritonitis virus [75].

CONCLUSION

While summing up the data presented in the paper, one should mention that typical feature of coronaviruses is a wide variety of their natural hosts along with the pronounced species-limited pathogenicity. Due to the capacity of infecting various organs the coronaviruses can be classified as pantropic viruses [1]. The diseases induced by them are mostly acute, but can transform to chronic and latent forms. All coronavirus species are specified by clear dependence of the disease severity on the age of the susceptible animal and intensity of the pathological processes. Thus, severe bronchitis was reported only in chicks (IBV), lethal hepatitis – in newborn mice (murine hepatitis virus), lethal pneumonia – in newborn rats (coronavirus of rats), severe lethal gastroenteritis and encephalomyelitis – only in piglets (TGEV and PHEV), lethal diarrhea – only in newborn calves (agent of coronavirus enteritis in calves). In adult animals of the same species the infection was subclinical and inapperant.

All described cases of the diseases induced by different coronaviruses have one thing in common – acute disorder of capillary circulation in the affected organ, which is the base of the pathological process caused by the agent. This results in the edematous organ, profuse serous exudate, and in the most pronounced cases – to necrosis and lining detachment. Similar signs were reported in case of "blue comb" disease in turkeys, when cyanosis of the comb and general asthenia were associated with severe enteritis and profuse diarrhea [14].

There is a definite amount of data on each species of coronaviruses described in the paper and these data allow for the comparative analysis of their properties, structure, antigenic relations as well as for getting an idea of their role in the pathology of farm and domestic animals and birds. However, even those pathological syndromes, which are currently reliably associated with the coronaviruses, are indicative of major damage to agriculture caused by these pathogens.

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Peer-review of study guide "Sheep pox and goat pox". A. V. Kononov, O. P. Byadovskaya, K. A. Shalina, A. V. Sprygin, V. I. Diev. Vladimir: FGBI "ARRIAH", 2021. 46 p.: fig. ISBN 978-5-900026-78-7

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Рецензия на учебное пособие «Оспа овец и оспа коз». А. В. Кононов, О. П. Бьядовская, К. А. Шалина, А. В. Спрыгин, В. И. Диев. Владимир: ФГБУ «ВНИИЗЖ», 2021. 46 с.: ил. ISBN 978-5-900026-78-7

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The study guide addresses two highly dangerous infectious transboundary diseases – sheep pox and goat pox. It particular, current literature data and the results of in-house research activities regarding sheep pox and goat pox epidemic situation in the Russian Federation and in the world are provided. Data on etiological structure and morphology of causative agents, sources of infection and transmission factors, pathogenesis patterns with indication of pathological process stages in infected animals, main clinical signs of the diseases and post-mortem lesions in sheep and goats are presented.

Considerable attention is given to the special aspects of sheep pox and goat pox diagnosis; methods for collection of biological material samples for virologic, serological, molecular genetic and histological tests are described in detail.

Attention is drawn to the necessity of differential diagnosis; the diseases and factors to be ruled out are indicated.

The study guide is extensively illustrated with figures showing specific clinical manifestations and post-mortem lesions in goat pox virus- and sheep pox virus-infected animals.

The study guide is targeted at students and trainees, in particular undergraduates, postgraduates, retrainees, and serves as the source of state-of-the-art knowledge on such significant infectious diseases as sheep pox and goat pox. Besides, the material will be helpful for the specialists of the state veterinary service and veterinary surveillance bodies of the country.

The authors undertook considerable work to analyze sheep pox and goat pox epidemic situation in the Russian Federation and countries around the world, as well as the existing measures to control and prevent the said diseases.

In my opinion, the publication of this study guide is timely, relevant and useful not only for students and trainees, but also for a wide range of veterinary professionals dealing with infections.

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Peer-review of study guide "African swine fever". A. S. Igolkin, K. N. Gruzdev. Vladimir: FGBI "ARRIAH", 2021. 126 p.: fig. ISBN 978-5-900026-79-4

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Рецензия на учебно-методическое пособие «Африканская чума свиней». А. С. Иголкин, К. Н. Груздев. Владимир: ФГБУ «ВНИИЗЖ», 2021. 126 с.: ил. ISBN 978-5-900026-79-4

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African swine fever (ASF) is the disease of wild and domestic pigs caused by the DNA virus of *Asfarviridae* family, *Asfivirus* genus. The virus strains and isolates are characterized by a considerable genetic and immunologic diversity, responsible for the differences in their biological properties. Since the ASF genotype II serotype 8 virus was introduced in our country (2007) this problem has been the most challenging one for the domestic pig production, causing significant economic losses to the whole industry and posing a serious threat to the national food security. In recent years, ASF virus has covered countries to the West and East from the Russian Federation, thus significantly expanding its habitat; and the disease itself brings multi-billion losses in many countries of the world. A potential simultaneous circulation of viral strains of different virulence levels makes it necessary to improve diagnostic and preventive measures in case of ASF suspicion, herewith the lack of specific prophylaxis and treatment means and insufficient knowledge complicate the control of this infection. Currently pursuant to the approved "Veterinary rules of application of preventive, diagnostic, restrictive and other measures, quarantine imposition and removal and other restrictions aimed at prevention of African swine fever spread and outbreak eradication" on a livestock farm or in a backyard, where an ASF outbreak is reported, the quarantine shall be imposed and all pigs shall be destroyed.

In recent years, the veterinary science and practice have built up broad experience in ASF prevention and outbreak eradication, though it is fragmented and requires generalization. This approach was used by the authors of the study guide under review "African Swine Fever" A. S. Igolkin and K. N. Gruzdev, leading ASF specialists.

The work under review consists of 11 sections and 126 pages. ASF epidemiology in Russia and other countries, disease etiology, ASFV genetic peculiarities, virus cultivation, isolate differentiation, its resistance to different chemical and physical factors and other issues are presented clearly in the study guide. The infection sources and transmission routes are analyzed. The modern methods of laboratory diagnosis, pathogenesis, clinical and post mortem signs of the disease as well as ASF prevention and control measures are described. Much attention is paid to ASF primary differential diagnosis, which is especially important for practicing veterinarians.

Materials given in the study guide reflect long-term experience of the authors and staff members of different research institutes. They will be useful for veterinary specialists of different levels in their practice. The study guide is easy to read, it is illustrated with high-quality informative figures and tables. Taking into account a high level of the presented material, the study guide might be recommended for use in educational processes and as an information resource.

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Рецензия на учебно-методическое пособие «Африканская чума свиней». А. С. Иголкин, К. Н. Груздев. Владимир: ФГБУ «ВНИИЗЖ», 2021. 126 с.: ил. ISBN 978-5-900026-79-4

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African Swine Fever (ASF) is a highly contagious, septicemia-causing disease of domestic pigs and wild boar. It poses a huge threat to the pig industry in all countries where these animals are reared. ASF is caused by a DNA virus of the *Asfivirus* genus of the *Asfarviridae* family. Global ASF fight experience has demonstrated that the absence of prophylactic and therapeutic drugs seriously hinders the disease control and eradication.

As practice shows, well-informed and well-trained people are required to implement preventive, diagnostic, restrictive measures aimed at ASF prevention and eradication.

The "African swine fever" study guide was prepared by leading experts of the scientific institution FGBI "ARRIAH" A. S. Igolkin, K. N. Gruzdev, who have rich practical experience in ASF control. The peer-reviewed work includes materials accumulated over the years on the infection etiology, genetic features of the ASF virus, principles of differentiation of isolates, virus replication, and virus resistance to various chemical and physical factors. The guide provides information on modern methods of the laboratory diagnosis, as well as on measures taken to prevent and control ASF.

The authors have described the world's experience gained in this sphere.

The theoretical and practical information covered in the study guide is equally important for the veterinary policy makers and for experts who directly take preventive, diagnostic and restrictive measures to prevent and eradicate ASF.

The chosen form of the study guide makes it possible to increase and consolidate knowledge about the issue in focus. The use of the self-control section helps to appropriately assess students' knowledge after they complete seminars, webinars, and courses (including the on-line ones).

The use of this study guide in the educational process will facilitate professional growth of veterinary practitioners.

It shall be noted that this is a well-illustrated and professional study guide with an extensive list of reliable reference materials used as a basis.

The presented study guide can be recommended for courses, seminars, advanced training webinars for veterinarians and biologists. It can also be used by municipal institutions, forest services, the Ministry of Emergency Situations and other people as a source of information about this socially important porcine disease.

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Фото: Александр Плонский

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Papers in two languages – Russian and English – that comprise results of own scientific studies, being up to 6–8 pages (up to 10 pages for reviews) but at least 5 pages (single-spaced, size 12) are accepted for publication. Optimal paper size: 3,000–6,000 words.

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РЕФЕРЕНТНАЯ ЛАБОРАТОРИЯ МЭБ ПО ВЫСОКОПАТОГЕННОМУ
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