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

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Nature of viruses and the radical change in viral taxonomy

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SUMMARY

A short report is devoted to the radical changes in the taxonomy of viruses. The metagenomic sequencing has revealed the presence of a vast variety of viruses in diverse environmental samples without any connections with banal parasitism, infectivity, or pathogenicity. The understanding of viruses has expanded beyond the original parasitic–pathogen model, and now virologists recognize the role of viruses in host regulation and the maintenance of natural ecosystems. Co-evolution of the viral and cellular genomes includes mutual horizontal gene transfer and joint development of new biological functions, such as the mechanism of phylogenesis and phylodynamics of coactants. The concepts of the origin of viruses and their relation to the Universal Tree of Life are formulated. In this regard, the International Committee on the Taxonomy of Viruses (ICTV) changed the previous Code of their classification hierarchy from five ranks to a fifteen-rank one, that emulates a Linnaean framework and accommodates the entire spectrum of genetic divergence in the virosphere. Changes in the rank hierarchy are based on the evolution of the recognition of virus taxa over time, from a traditional phenotype-based characterization process to a multistage process that includes comparative sequence analyses of conserved genes and proteins, including gene phylogeny, gene synteny and shared gene content. The ICTV, that oversees the official classification of viruses and nomenclature of taxa, accepts possible non-hierarchical classifications of viruses beyond taxonomic attribution. The above provisions are illustrated with schemes of chimeric polyphyletic origin of viruses and a new rank structure; the table gives examples of the modern classification of viruses that cause some socially significant infections.

Keywords: review, viruses, taxonomy

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О природе вирусов и радикальном изменении их таксономии

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РЕЗЮМЕ

Краткое сообщение посвящено радикальным изменениям в таксономии вирусов. С помощью метагеномного анализа установлено существование огромного количества разнообразных вирусов в окружающей среде во всех сферах жизни без каких-либо связей с тривиальными паразитизмом, инфекционностью, патогенностью. Представление о вирусах вышло за рамки первоначальной модели паразитических патогенов с признанием их роли в биологии хозяина и поддержании естественных экосистем. Коэволюция вирусного и клеточного геномов включает взаимный горизонтальный перенос генов и совместное создание новых биологических функций как механизм филогенеза и филодинамики соактантов. Сформулированы представления о происхождении вирусов и их отношении ко Всеобщему Древу Жизни. В связи с этим Международный комитет по таксономии вирусов изменил прежний код их классификационной иерархии из пяти рангов на пятнадцатиранговый, тесно согласующийся с современной линнеевской таксономией и вмещающий в систему органического мира весь спектр генетического многообразия виросферы. Изменения в ранговой иерархии таксонов базируются

на прогрессе от традиционной сегрегации вирусов на основе фенотипа до многоступенчатого процесса, включающего сравнительные характеристики последовательностей консервативных генов и белков, филогению генов, их синтез, общее содержание. Международный комитет по таксономии вирусов, осуществляющий надзор за официальной классификацией вирусов и номенклатурой таксонов, допускает возможные неиерархические классификации вирусов вне таксономической компетенции. Приведенные положения иллюстрированы схемами химерного полифилетического происхождения вирусов и новой ранговой структуры, в таблице даны примеры современной классификации вирусов – возбудителей некоторых социально значимых инфекций.

Ключевые слова: обзор, вирусы, таксономия

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Viruses in the biosphere are ubiquitous and as obligate intracellular parasites infect organisms of all biological species, including giant viruses. Therefore, they are probably the most abundant organism on the planet, forming a specific community of living matter of an independent type, defined as the virosphere. Based on this, the original principle of dividing biological entities into two groups of organisms is defined: ribosomal organisms, which include eukaryotes, archaea, and bacteria, and capsid organisms, which include viruses. For other self-replicating structures, the term 'orphan replicons' has been proposed (plasmids and viroids) [1, 2].

Up to some moment, science and practice were focused mainly on the negative effects of viral existence, based on the definition given by Andre Lwoff (1957) when formulating the concept of viruses as «strictly intracellular and potentially pathogenic entities, with an infectious phase, and possessing only one type of nucleic acid, multiplying in the form of their genetic material, unable to grow and to undergo binary fission and devoid of a "Lipmann system" (i.e. a system of enzymes for the production of energy)» [3]. In the modern view, virus is a capsid-encoding organism that is composed of proteins and nucleic acids, self-assembles in a nucleocapsid and uses a ribosome-encoding organism for the completion of its life cycle [4, 5].

Recent virological studies of the biosphere, their fundamental and applied results have become a new impetus for the development of virology as a science of a peculiar form of life, living matter at one of the initial stages of its origin and evolution, previously united into a separate kingdom of *Vira*. Hundreds of thousands of predominantly unknown giant deoxyriboviruses forming the marine viral metagenome (or virome), mainly of *Prochlorococcus* bacteria – the main element of photosynthesis on Earth, suggested a different role of viruses in the biosphere, in the planetary cycle of genes, matter, energy, in the economy of nature in general. They are responsible for the extraordinary abundance of living creatures in an important, if not the most important part of the planet – the World Ocean, the primary source of living matter. Under these conditions, detritus, the products of viral-induced lysis of plankton single-celled organisms, provides more than a third of organic resources in aquatic ecosystems; a bypass, simplifying and accelerating vital processes viral shunt is formed, giving a total annual yield of about 0.5 gigatons of carbon [6]. Herewith, the density of the viral population in the surface layers of the ocean is apparently estimated in hundreds of millions of viral particles per liter of water [7].

The data obtained during the study of new elements of virology allowed us to suggest a hypothesis regarding the role of virogenesis in the development of cellular life forms. The structure and biochemistry of the newly discovered largest mimiviruses have much in common with the eukaryote nucleus and suggest possible viral eukaryogenesis by analogy with the vector of endosymbiotic origin of other important cellular organelles, in particular marine free-living alphaproteobacteria → mitochondria, cyanobacteria → chloroplasts of fungi and plants [4, 8].

However, conceptually, the attribution of viruses to the living world is still questioned by many, and living cells, *de facto* hosts in viral parasitic systems, are considered to be a kind of reaction medium for the metabolic realization of alien genetic information. In particular, one of the founders of Russian virology, Academician V. M. Zhdanov (1914–1987), denied the existence of viruses on the population level, giving the preference exclusively to biosynthetic aspects in research. The strict separation between living and non-living beings puts viruses far from the Universal Tree of Life and, in fact, excludes them from the modern system of the organic world, although objectively they play an important role in evolution – the force that drives the development of all life on Earth.

Advances in genomic sequencing and comparative genomics over the past decade have made it possible to elucidate many elements of the evolutionary relationships between organisms at the subcellular and cellular levels. The metagenomic sequencing has revealed the presence of a vast variety of viruses in diverse environmental from apparently healthy environment samples without any connections with banal parasitism, infectivity, and pathogenicity.

The understanding of viruses has expanded beyond the original parasitic-pathogen model, and now virologists recognize the role of viruses in host regulation and

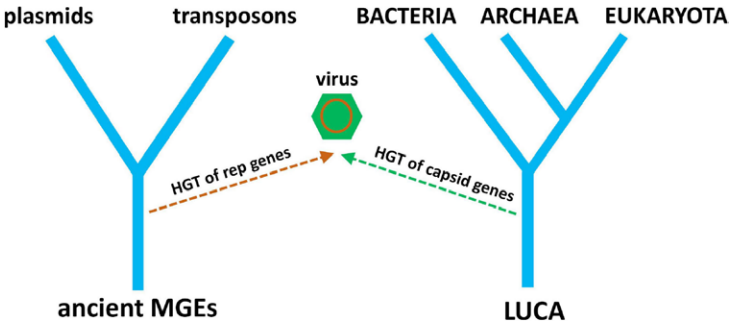

Fig. 1. A simple tree diagram showing the chimeric origin of viruses from pre-LUCA replication genes and post-LUCA structural genes. Ancient MGE ancestors replace ancient cells, reflecting the origin of virus replication genes from MGEs. The evolution of modern plasmids and transposons from ancient MGEs is also depicted [10]

the maintenance of natural ecosystems [9]. In particular, co-evolution of the viral and cellular genomes includes mutual horizontal gene transfer and joint development of new biological functions, such as existent and widely "used" mechanism of phylogenesis and phylodynamics of coactants. From the tiny circoviruses, 12–27 nm in size, harboring only two genes replicated and transcribed by cellular enzymes having one structural protein to novel giant deoxyriboviruses 400–800 nm in size, containing 1,200 b.p. genome, which encodes more than 900 polypeptides; viruses and their host cells are ecologically and evolutionarily intertwined [10].

The following diagram defining the origin of viruses and their relation to the Universal Tree of Life can serve as a kind of summarizing evidence (Fig. 1).

Virus taxonomy emerged as a discipline in the middle of the twentieth century. Traditionally, classification by virus taxonomists has been focused on the grouping of evidently closely related viruses. The earliest version, the nomenclature of viruses often connected to their 'micro-evolution' and recognizing only genera and families, has developed over time into a five-rank hierarchy of species, genus, subfamily, family and order based on Linnaean system of classification, which remained in place until 2017. However, today taxonomy as a branch science in biology, studying the principles, methods and rules of organism classification, is one of the developing areas, deploying more and more novel methods of mathematical statistics and computational biology, computer analysis, DNA and RNA comparative analysis, analysis of cell ultrastructure and many other possibilities [11].

In this regard during the past few years, the International Committee on Taxonomy of Viruses (ICTV) has recognized that the taxonomy it develops can be usefully extended to include the basal evolutionary relationships among distantly related viruses. Consequently, the ICTV has changed its 5-rank code to allow a 15-rank classification hierarchy that closely aligns with the Linnaean taxonomic system and may accommodate the entire spectrum of genetic divergence in the virosphere (Fig. 2) [12].

The novel structure includes eight principal (or primary) ranks and seven derivative (or secondary) ranks. The eight principal ranks include four that were already in use (*species*, *genus*, *family*, and *order*), and four that are new (*class*, *phylum*, *kingdom*, *realm*). Six new ranks are derivatives of most of the remaining principal ranks. This nomenclature of principal and derivative ranks includes defined suffixes for taxa, follow those used in the Linnaean system.

Changes in the rank hierarchy are based on the evolution of the recognition of virus taxa over time, from a traditional phenotype-based characterization process to a multistage process that includes comparative sequence analyses of conserved genes and proteins, including gene phylogeny, gene synteny, shared gene content and other molecular features [12]. To illustrate the adopted changes, the table shows the hierarchical position of some current viruses.

As follows from the above examples, the divergence of viruses progressively increases within the hierarchical cluster from the basal (realm) to the apical (species) rank (compare representatives of the *Riboviria* realm). Not all derivative ranks are fully populated by the

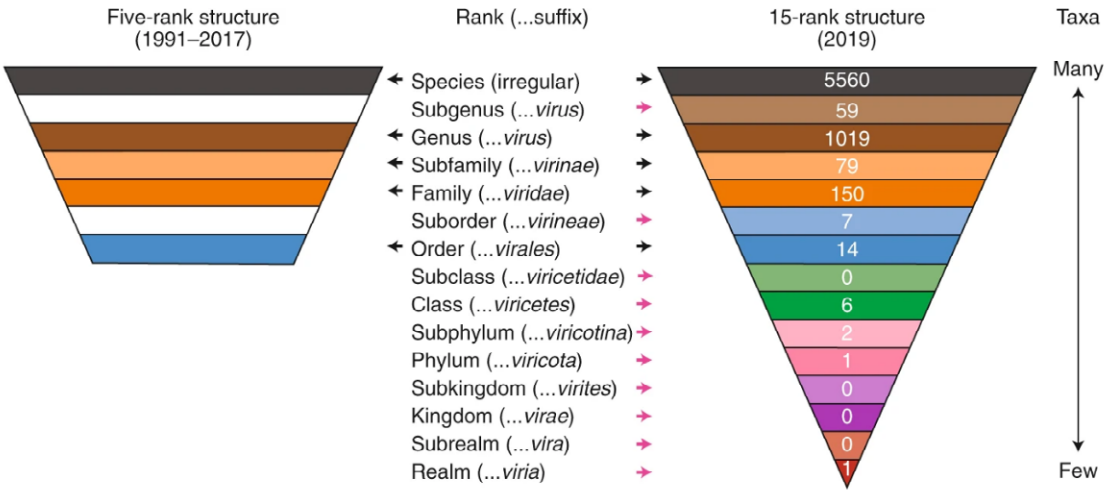

Fig. 2. The previous and the new taxonomic ranks and the number of taxa (shown in white font on the 15-rank structure). Black arrows mean ranks common to both structures; pink arrows denote ranks introduced in the 15-rank structure [12]

viruses (compare African swine fever, avian influenza and SARS-CoV-2 viruses). There are many unresolved issues due to the insufficient characteristics of macroevolutionary ranking of individual viruses, so their taxonomy, as well as in the whole organic world, is a dynamic and continuous process.

In particular, the family *Asfarviridae* is assigned to the order *Asfuvirales* of the *Pokkesviricetes* class and ranks higher (see Table). However, in addition to the official nomenclature the inclusion of the family of the unique African swine fever virus in the assumed order *Megavirales*, composed of members of a monophyletic, but heterogeneous lineage of large nuclear-cytoplasmic deoxyriboviruses, formerly called giruses, which also included the poxviruses, iridoviruses, phycodnaviruses, mimiviruses and other members of similar families, is still discussed [1]. With the constant discovery of new giant viruses (pandoroviruses, faustoviruses, molliviruses, etc.), this group is likely to increase in the near future and the taxonomy of its members remains debatable [13].

The International Committee on the Taxonomy of Viruses is a committee of the Virology Division of the International Union of Microbiological Societies (IUMS), which supervises the official classification of viruses and the nomenclature of taxa. Since ICTV is a voluntary, largely self-regulating and non-profit global organization, it allows, in addition to the official taxonomy, possible classifications of viruses outside the taxonomic competence of the ICTV.

For example, a non-hierarchical classification of viruses by D. Baltimore groups viruses into just seven classes according to their genome type, and the way their genetic information is realized, is generally recognized and widely used. The classification takes into consideration whether the DNA viruses contain double-stranded or single-stranded genome or replicate through single-stranded RNA; and RNA viruses containing positive or negative double or single-stranded genome or whether they replicate by reverse transcription [14]. For practical epidemiology, the real phylodynamic systematics of viruses based on parasite systems and other biological system approaches with distribution into such ecological categories as reservoirs, amplifiers, sources of infection, transmission is certainly important. Among other existing and possible non-hierarchical classifications, systematization techniques based on models of viral morphogenesis and morphology would be extremely interesting and useful, where the nature and mechanisms of formation of icosahedral capsid structures have practical significance from the point of view of virus resistance outside the body.

Table
Current classification of the viruses causing some infections of social importance in the 15-rank taxonomic hierarchy [12]

Ranks	Viruses					
	African swine fever	Bovine leukemia	Avian influenza	Rabies	FMD	SARS-CoV-2
Species	*African swine fever virus*	*Bovine leukemia virus**	*Influenza A virus***	*Rabies lyssavirus****	*Foot-and-mouth-disease virus*	*Severe acute respiratory syndrome-related coronavirus*****
Subgenus	–	–	–	–	–	*Sarbecovirus*
Genus	*Asfivirus*	*Deltaretrovirus*	*Alphainfluenzavirus*	*Lyssavirus*	*Aphthovirus*	*Betacoronavirus*
Subfamily	–	–	–	–	–	*Orthocoronavirinae*
Family	*Asfarviridae*	*Retroviridae*	*Orthomyxoviridae*	*Rhabdoviridae*	*Picornaviridae*	*Coronaviridae*
Suborder	–	–	–	–	–	*Cornidovirineae*
Order	*Asfuvirales*	*Ortervirales*	*Articulavirales*	*Mononegavirales*	*Picornavirales*	*Nidovirales*
Subclass	–	–	–	–	–	–
Class	*Pokkesviricetes*	*Revtraviricetes*	*Insthoviricetes*	*Monjiviricetes*	*Pisoniviricetes*	*Pisoniviricetes*
Subphylum	–	–	*Polyploviricotina*	*Haploviricotina*	–	–
Phylum	*Nucleocytoviricota*	*Artverviricota*	*Negarnaviricota*	*Negarnaviricota*	*Pisuviricota*	*Pisuviricota*
Subkingdom	–	–	–	–	–	–
Kingdom	*Bamfordvirae*	*Pararnavirae*	*Orthornavirae*	*Orthornavirae*	*Orthornavirae*	*Orthornavirae*
Subrealm	–	–	–	–	–	–
Realm	*Varidnaviria*	*Riboviria*	*Riboviria*	*Riboviria*	*Riboviria*	*Riboviria*

* synonyms *Bovine leucosis virus*, *Bovine type C oncovirus*;
***serotype Influenza A virus subtype H5N1*;
*** members *Rabies virus*, *Arctic rabies virus*;
**** no rank *SARS-CoV-2*.

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More on search for causes of sensitization to tuberculin PPD for mammals in cattle

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SUMMARY

Despite the large number of papers dealing with the description of proposed methods for bovine tuberculosis diagnosis and mechanisms of non-specific reaction development in diseased and healthy animals, various aspects require further study. Many specialists are still of the view, formulated when studying causes of pseudoallergic reactions, that the agents of actinomycosis, trematode infections etc. can cause sensitization of the animal body to tuberculin PPD for mammals. The possibility of sensitization of cattle body to *Actinomyces bovis* tuberculin was studied in 240 animals identified as actinomycosis diseased among 3,473 tested animals. Only 11 (4.6%) of the total number of diseased animals were reactors to tuberculin PPD for mammals. During bacteriological tests of material from animals euthanized for diagnostic purposes (tuberculin reactors and nonreactors with a confirmed postmortem diagnosis of actinomycosis), acid-tolerant nontuberculous mycobacterium (NTM) cultures were isolated. The results of the experiment conducted in 628 cows of a dairy holding confirmed that *Actinomyces bovis* lacks tuberculin-associated allergenicity: actinomycosis was detected only in one of 96 (15.2%) tuberculin reactors. The conducted clinical tests with high significance level ($P < 0.005$) showed that there is no association between allergic reaction to tuberculin PPD for mammals and actinomycosis infection. The obtained results are indicative of imperfections in differential diagnosis, and further studies in this field should significantly contribute to gaining a better insight into non-specific sensitization of cattle body to tuberculin.

Keywords: tuberculosis, actinomycosis, sensitization, diagnosis, differentiation, parallergy, pseudoallergic reactions, mycobacteria, atypical acid-tolerant mycobacteria

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К поиску причин сенсибилизации крупного рогатого скота к ППД-туберкулину для млекопитающих

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РЕЗЮМЕ

Несмотря на большое количество работ, посвященных характеристике предложенных методов диагностики туберкулеза крупного рогатого скота и механизмам проявления неспецифических реакций у больных и здоровых животных, многие аспекты этой проблемы требуют дополнительного изучения. До настоящего времени многие специалисты придерживаются точки зрения, сформулированной при изучении причин псевдоаллергических реакций, согласно которой возбудители актиномикозной инфекции, трематодозной инвазии и др. могут быть причиной сенсибилизации макроорганизма животных к ППД-туберкулину для млекопитающих. Возможность сенсибилизации организма крупного рогатого скота к туберкулину *Actinomyces bovis* изучали

на 240 больных актиномикозом животных из 3473 исследованных. Из числа больных только 11 голов (4,6%) реагировали на ППД-туберкулин для млекопитающих. При бактериологическом исследовании материала от убитых с диагностической целью животных (реагировавших и не реагировавших на туберкулин с подтвержденным на актиномикоз патолого-анатомическим диагнозом) изолированы культуры кислотоустойчивых нетуберкулезных микобактерий. Отсутствие аллергизирующих к туберкулину свойств у *Actinomyces bovis* было подтверждено результатами эксперимента, проведенного на 628 животных одного из молочных комплексов, где только у одной из 96 (15,2%) реагирующих на туберкулин коров выявили актиномикоз. Проведенные клинические исследования с высокой степенью достоверности ($P < 0,005$) позволили установить отсутствие взаимосвязи между аллергией на ППД-туберкулин для млекопитающих и актиномикозным инфекционным процессом. Полученные результаты свидетельствуют о несовершенстве дифференциальной диагностики, и дальнейшие исследования в этой области должны внести существенный вклад в развитие представлений о неспецифической сенсибилизации организма крупного рогатого скота к туберкулину.

Ключевые слова: туберкулез, актиномикоз, сенсибилизация, диагностика, дифференциация, парааллергия, псевдоаллергические реакции, микобактерии, атипичные кислотоустойчивые микобактерии

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INTRODUCTION

Non-specific reactions to tuberculin PPD for mammals constitute an issue that affects animal husbandry worldwide and has not been fully resolved despite the numerous differentiation methods proposed. According to official statistics, including those from the World Organisation for Animal Health, at present, tuberculin reactors make up about 54% of tested animals globally, in the CIS member states – from 12.6 to 62.4%, in the Subjects of the Russian Federation – from 7.8 to 49.3% [1–4].

Amongst the variety of microbial biota causing sensitization of the animal body to tuberculin, atypical acid-tolerant mycobacteria of groups II, III and IV (according to the classification of E. Runyon) are most significant as regards the number and levels of the caused skin reactions. Acid resistant saprophytes can also play a significant role in the allergization of the body [3, 5–10].

According to current conceptions, microoganisms that are closely related to mycobacteria, namely corynebacteria, *Nocardia* and rhodococci, also have tuberculin sensitizing properties. Their essential characteristics are genus and species specificity to mycobacteria, as well as common group specific antigens detected when cross reactivity occurs. Interest in these microorganisms has at present expanded due to their more frequent isolation from the biomaterial from tuberculin reactive animals. Experimental and clinical data show that corynebacterium cultures are isolated in 28.6% of cases [6, 11, 12], *Nocardia* cultures – in 16.9% of cases [12], and rhodococci – in 26.3% of cases [11, 13–15].

There is no doubt that parallergies hold a key position among the causes of non-specific sensitization of the animal body. At the same time, characteristic reactions can be attributable to other causes not associated with mycobacteria, various purulent processes caused by transient microorganisms, parasitic diseases (trematode infections), stress factors, protein load [3, 4, 9, 16].

Allergy is also attributed to different pregnancy pathologies, animal diets with an unbalanced proportion of micro- and macronutrients, feeding with mold fungus infected feedstuffs, etc. [16–18].

However, the further studies of allergic reactions to tuberculin in animals affected with trichophytosis, dicroceliosis, paramphistomatosis, echinococcosis did not reveal any correlation. No association is found between sensitization to tuberculin and stress factors, in particular in cows in the antepartum and postpartum periods [19–21].

Convincing experimental evidence was provided with respect to the absence of allergy in animals receiving urea [16, 22].

The comparison between the results of coprological tests of samples collected from fascioliasis affected animals and intradermal tuberculin test results yielded mutually exclusive data. Tuberculin reactors made up 5.8% of the tested fascioliasis affected animals, the coprological test results were negative in 22% of tuberculin reactive animals [5, 7].

The interest in examining the possibility of animal body sensitization to tuberculin by microorganisms having common genus specific characteristics with mycobacteria, in particular A*ctinomyces bovis* of the genus *Actinomyces* of the order *Actinomycetales* with characteristic high population genetic homogeneity with the genus *Mycobacterium*, has at present expanded [2, 3, 11, 16, 18, 23].

Many specialists are currently of the view, formulated when studying the genetic characteristics of these microorganisms, that evolutionary commonality and possible presence of common antigens can be responsible for sensitization to tuberculin [24, 25].

Table 1
Distribution of tuberculin reactor animals by actynomycosis occurrence level

No.	Farm	Number of tested animals	Intradermal tuberculin test reactors		Actinomycosis diseased cattle		Out of these			
							tuberculin reactors		tuberculin nonreactors	
			number of animals	%	number of animals	%	number of animals	%	number of animals	%
1	SPK "Ordzhonikidze"	740	–	–	43	5.8	–	–	43	100.0
2	SPK "Lenina"	859	78	9.1	45	5.2	2	4.4	43	95.6
3	KFKh "Paraulsky"	300	8	2.7	19	6.3	1	5.3	18	94.7
4	SPK "Buynaksky"	500	66	13.2	69	13.8	5	7.2	64	92.8
5	SPK "Gelinsky"	300	48	16.0	59	19.7	3	5.1	56	94.9
6	KFKh "Rassvet"	543	–	–	4	0.7	–	–	4	100.0
7	KFKh "Tavrida"	231	–	–	1	0.4	–	–	1	100.0
	Total	3,473	232	6.7	240	6.9	11	4.6	229	95.4

According to the data from some clinical studies, actinomycosis diseased animals are reactive to tuberculin for mammals. However, no experimentally proven and laboratory-confirmed study results have been found.

The purpose of the study was to substantiate experimentally a possible association between reactions to tuberculin tests and actinomycosis infection.

MATERIALS AND METHODS

Clinical tests were carried out in 3,473 cattle of different age and sex groups on seven farms of the Karabudakhkentsky and Novolaksky Raions of the Republic of Dagestan.

Tests for assessment of tuberculin sensitizing properties of *Actinomyces bovis* were carried out in 628 cattle at a dairy complex located in the Babayurtovsky Raion of the Republic of Dagestan.

Allergy tests of cattle were carried out in accordance with the "Veterinary rules for implementation of preventive, diagnostic, restrictive and other measures, imposition and lifting of quarantine and other restrictions aimed to prevent the spread and eradicate the outbreaks of tuberculosis" approved by Order of the Ministry of Agriculture of the Russian Federation No. 534 of 08 September 2020[1].

The identification of the isolated mycobacterium cultures was performed according to GOST 26072-89 "Agricultural animals and poultry. Methods of laboratory diagnostics of tuberculosis"[2] and GOST 27318-87 "Agricultural animals. Methods of identification of atypical mycobacteria"[3].

The animals were subjected to clinical examination for actinomycosis, including palpation of the affected area; laboratory tests of tissues collected from the infected areas were carried out. The diagnosis was considered to be confirmed only when *Actinomyces bovis* was isolated from the material. In case of detection of neoplasms of unknown etiology, histological analysis was performed in accordance with the generally accepted procedures.

The obtained data were statistically processed using parametric techniques; statistical significance of the results was determined with Student's test.

TEST RESULTS

Allergy tests carried out on the farms revealed non-specific sensitization of animals to tuberculin PPD for mammals. The percentage of reactor animals varied from 2.7 to 16.0%. Actinomycosis diseased animals made up from 0.4 to 19.7% (Table 1).

The tests showed that 232 (6.7%) out of 3,473 animals reacted to intradermal tuberculin tests, the number of actinomycosis diseased animals amounted to 240 (6.9%).

Among actinomycosis diseased cattle, only 11 animals (4.6%) tested tuberculin PPD positive, 229 animals (95.4%) were nonreactors.

On the farm SPK "Ordzhonikidze", only 43 animals (5.8%) out of 740 tested cattle were actinomycosis diseased, no tuberculin reactors were detected. Similar results were obtained for the small scale farms KFKh "Rassvet" and KFKh "Tavrida" located in the Karabudakhkentsky Raion.

The following actinomycosis diseased animals were euthanized and subjected to postmortem examination: 11 tuberculin reactors and 10 animals with negative allergy test results (Table 2).

As a result of the tests performed, *Mycobacterium scrofulaceum* culture was isolated from the biomaterial from tuberculin positive animals, 7 strains were identified (63.6%). This indicates that the sensitization of the body was caused

[1] https://docs.cntd.ru/document/565721619.
[2] https://docs.cntd.ru/document/1200025492.
[3] https://base.garant.ru/5917269.

Table 2
The results of postmortem examination and bacteriological tests of biomaterial from actinomycosis diseased animals

Actinomycosis diseased animals	Number of animals	Post-mortem diagnosis	Bacteriological test results
Tuberculin reactors	11	actinomycosis	7 *Mycobacterium scrofulaceum* strains were isolated
Tuberculin nonreactors	10	actinomycosis	2 *Mycobacterium scrofulaceum* strains and 1 *Mycobacterium phlei* strain were isolated

by atypical mycobacteria. The true cause of sensitization was not identified for 4 animals, and this is indirectly indicative of imperfections of the proposed methods for tuberculosis diagnosis in animals, in this particular case – of laboratory methods. This suggests that sensitization can be due to the presence of hydrocarbon oxidizing microorganisms (corynebacteria, *Nocardia*, rhodococci, etc.) in the cattle body, the isolation of which requires specific test procedures.

On the other hand, the isolation of two *Mycobacterium scrofulaceum* strains and one strain of saprophytic mycobacteria (*Mycobacterium phlei*) from the biomaterial samples from animals with negative allergy test results should be considered as confirmatory evidence for the existing view that atypical mycobacteria are not always able to cause the sensitization of the animal body to tuberculin.

Extensive literature data, as well as the results of the study show that acid-tolerant nontuberculous mycobacteria are able to persist in the body latently, without manifesting themselves in any way, for long periods of time; this explains negative laboratory test results in 8 of 10 animals.

Tests for assessment of tuberculin sensitizing properties of *Actinomyces bovis* were carried out in 628 cattle at a dairy complex located in the Babayurtovsky Raion of the Republic of Dagestan. Among the tested cattle, 96 animals (15.2%) were tuberculin reactors, no actinomycosis diseased animals were detected.

Based on the comparison of clinical signs and data from the laboratory tests of animals for actinomycosis with allergy test results, it can be concluded that the obtained results show with high significance level ($P < 0.005$) that there is no correlation between actinomycosis infection and allergic reactions to tuberculin.

DISCUSSION AND CONCLUSION

The test results are consistent with extensive literature data on the studies of specificity of tuberculin PPD for mammals that demonstrate apparent specificity of the allergen towards homologous sensitization.

Less intense in manifestation, short term reactions to tuberculin are reported in animals infected with acid-tolerant nontuberculous mycobacteria (NTM), as well as with mycobacterium-like (hydrocarbon oxidizing) microorganisms (corynebacteria, *Nocardia*, rhodococci, etc.).

According to the statistics available, cross-reactions are often detected, and this is indicative of the high degree of the structural homogeneity of antigens, hence the need for using more effective differential diagnosis methods (postmortem examination, bacteriological testing).

It is important to note that, in some cases, negative laboratory test results call into question the effectiveness of the diagnostic method applied, since mycobacteria are characterized by continuous variation (in the opinion of the majority of researchers, owing to antigen drift), which results in new serological pathogenic variants.

In view of this, as well as taking into account culture method imperfections, a complex of diagnostic methods, in particular molecular genetic techniques (ELISA, PCR, etc.), should be used for isolation of commonly occurring transformed forms of mycobacteria (L-forms, spheroplasts, protoplasts, filterable forms, etc.), as well as for detection of latently persistent forms.

In the light of the obtained unequivocal evidence presented in this paper, when identifying the causes of pseudoallergic reactions, one should give due consideration to the fact that actinomycosis and parasitic infections are characterized by mixed clinical signs. In particular, atypical mycobacteria (*Mycobacterium* subsp.) and mycobacterium-like microorganisms (*Corynebacterium*, *Nocardia* and *Rhodococcus* subsp.), being typical representatives of gastrointestinal microbiota, can cause sensitization of the body to tuberculin in case of immunity status decline in animals.

The results of the study are consistent with the previously obtained data on identification of association between reactions to tuberculin and invasive diseases (trichophytosis, echinococcosis, dicroceliosis, fascioliasis, etc.) that show the absence of statistically significant correlation between them.

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Semi-quantitative risk assessment of peste des petits ruminants introduction with wild animals into Russian Federation

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SUMMARY
The Russian Federation was officially recognized free from peste des petits ruminants (PPR). As far as the disease infects both domestic and wild small ruminants, it is important to identify the level of the threat associated with the wild fauna diversity in the neighboring countries, where PPR outbreaks were reported. For that reason, habitats of various disease susceptible animal species were examined. Habitats of the wild susceptible animals were mapped for further examination of the interactions between different animal species using zoological research data; PPR outbreaks in wild animals were also designated in the map thus allowing for the detection of the potential routes of the infection spread in the population and introduction to the country. Analysis of the PPR epidemic situation in the country demonstrated that the disease cases were reported in wild mountain animals (ibices and moufflons) and migratory steppe animals (gazelles and saigas). Risk of this highly contagious viral disease spread in wild small ruminants in Mongolia was reported (probability 0.77). Expert survey was carried out for the determination of possible trends and factors of the infection introduction with the wild susceptible animals, through which small ruminant epizootologists assessed the risk probability. During the survey it was determined that PPR was expected to be introduced from Mongolia (probability 0.81), and of major significance were seasonal migrations of wild animal populations. The resulted semi-quantitative parameters of the potential risk can be recommended for the arrangement and implementation of measures aimed at prevention of PPR introduction and spread in the intact domestic and wild small ruminant populations inhabiting the territory of the Russian Federation.

Keywords: peste des petits ruminants (PPR), analysis, wild small ruminants, risk, disease spread, epidemic situation, PPR introduction into the Russian Federation

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Полуколичественная оценка риска заноса чумы мелких жвачных на территорию Российской Федерации с дикими восприимчивыми животными

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РЕЗЮМЕ

Российская Федерация признана страной, благополучной по чуме мелких жвачных. Поскольку данное заболевание поражает как домашних, так и диких мелких жвачных, необходимо оценить уровень угрозы, связанный с разнообразием дикой фауны в граничащих с Россией странах, в которых зарегистрированы вспышки чумы мелких жвачных. Для этого было проведено исследование ареалов обитания различных видов диких животных, восприимчивых к данному заболеванию. С целью изучения взаимодействия популяций различных видов животных с использованием данных зоологических исследований была составлена карта ареалов обитания диких восприимчивых животных, на которой также отмечены вспышки чумы среди диких животных, позволяющие выявить существующую возможность распространения инфекции внутри этих популяций и заноса инфекции на территорию страны. В ходе анализа эпизоотической ситуации по чуме мелких жвачных установлено, что в приграничных государствах зафиксированы случаи заболевания диких горных (горные козлы и бараны) и степных мигрирующих (джейраны и сайгаки) животных. Выявлена опасность распространения данной высококонтагиозной вирусной болезни среди диких мелких жвачных Монголии (вероятность реализации события 0,77). При определении возможных направлений и факторов заноса инфекции с дикими восприимчивыми жвачными был проведен экспертный опрос, в котором специалисты в области эпизоотологии болезней мелкого рогатого скота оценили вероятность реализации опасности. В ходе опроса было установлено, что ожидается занос чумы мелких жвачных в Российскую Федерацию со стороны Монголии (вероятность 0,81), а самым значимым фактором являются сезонные миграции популяций диких жвачных. Полученные полуколичественные показатели уровня вероятной угрозы можно рекомендовать к использованию при планировании и реализации мер по недопущению заноса и распространения чумы мелких жвачных в интактные популяции домашнего и дикого мелкого рогатого скота на территории Российской Федерации.

Ключевые слова: чума мелких жвачных, анализ, дикие мелкие жвачные, риск распространения заболевания, эпизоотическая ситуация, занос чумы мелких жвачных на территорию РФ

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INTRODUCTION

Peste des petits ruminants (PPR) known as goat plague and stomatitis-pneuma enteritis syndrome, is an infectious viral disease of domestic and wild small ruminants which poses threat to food security and balanced recourses in Africa, Middle East and Asia.

PPR outbreaks have been reported in the disease-free regions of the world and cause economic losses [1].

The recent reports and reviews on the PPR cases demonstrate the ongoing geographic expansion of the nosoarea in non-vaccinated susceptible populations of domestic small ruminants which contributes to there virus spread in areas where domestic animals and other species of susceptible species coexist. Thus, PPR caused high morbidity and mortality of the Mongolian saiga (*Saiga mongolica*), resulting in 80% decrease in the population and threatening with this subspecies extinction. The PPR clinical signs were also registered in other wild cloven-hoofed animals in Asia. In Pakistan PPR was detected in Sindh ibex (*Capra aegagrus blythi*). Recent PPR outbreaks were detected in China in Siberian ibex (*Capra ibex sibirica*), argali (*Ovis ammon*) and goitered gazelle (*Gazella subgutturosa*) [2–10].

In several PPR endemic countries there is more and more serological evidence of PPR reinfection in different wild animal species but the disease in these populations has not been confirmed [11, 12].

The wild fauna involvement in PPR spread is still understudied, however, the role of domestic small ruminants as the source of infection for wild fauna is obvious. Wild small ruminants are commonly found in PPR affected countries and inhabit most of the pastures together with small ruminants. It indicates that wild susceptible animals have a great potential for the disease transmission both between the populations of wild animals and between wild and domestic animals [13].

This fact becomes especially critical for the border regions of the Russian Federation with high density of the small ruminant population when the epidemic situation in neighboring countries is tense [14] and susceptible wild animal species are diverse.

The aim of the research was to determine the potential for PPR spread by susceptible wild animal populations in the RF border regions with subsequent assessment of the risks of the disease introduction into the territory of our country with wild small ruminants.

MATERIAL AND METHODS

In the framework of risk assessment of PPR introduction to the RF with wild ruminants the habitat of wild animal species susceptible to this disease as well as their diurnal activity were studied. PPR outbreaks in target species populations were taken into account. Estimates (score)

reflecting the potential for the infection spread between the populations were determined. To study the interrelation between the populations of different animal species using the data of zoological tests a map demonstrating habitats of wild susceptible animals was generated. The map shows PPR outbreaks in wild animals allowing to detect the possibility of PPR spread within these populations and the infection introduction to the RF territory. The cartographic materials were compiled using ArcGIS program.

There were determined three factors capable of influencing the potential for the infection spread between the populations: different populations inhabiting common territory (a), PPR outbreaks in wild fauna (b) diurnal activities (c). To determine the significance of scores each estimate was characterized (Table 1).

The estimates were assigned to wild animal species (Table 2) and the arithmetic mean of these estimates was calculated. Then the average estimates were converted to a fraction of the maximum possible score. Thus, the capability of the infection to spread by the wild ruminant populations was determined (Table 3). The data obtained were rated according to the risk level.

After the major directions of PPR introduction with wild ruminants were determined, a survey was conducted in the framework of which the experts in the field of epizootology and small ruminant diseases assessed the probability of the disease introduction to the Russian Federation and gave scores from 1 to 5, where 1 – the least probability, and 5 – the greatest probability of the event occurrence. Due to the survey it was possible to identify the most probable factor, as well as the direction of the introduction of PPR into the country.

Analysis of expert opinions (Table 4) was performed according to the following algorithm [16]:

X*i*, *j* – average estimates of the factors of PPR introduction to the RF under study;

Y*i* – the value of the probability of PPR introduction into the RF.

Herewith, the risk value for Y*i* ranged from 0 to 1, that's why the probability of introduction was calculated according to the formula:

$$(\sum Yi / X) / R,$$

where $\sum Yi$ – the sum of estimates X*i*, *j*;

X – the maximum number of scores for three factors [12];

R – maximum risk.

In all the tests the risk level was rated on the scale [17, 18]: $R \leq 0.3$ – negligible risk of the event occurrence; $0.3 < R \leq 0.5$ – low risk of the event occurrence; $0.5 < R \leq 0.7$ – moderate risk of occurrence; $0.7 < R \leq 0.9$ – high risk of the event occurrence.

RESULTS AND DISCUSSION

The Russian Federation is recognized free from PPR. In order to prevent the infection introduction and spread into the area with the naïve small ruminant population and to maintain the disease free status the risk of the infection introduction with wild fauna was assessed.

The OIE Terrestrial Code recommends to asses the risks of the infectious disease introduction by detection and analysis of the factors facilitating the infection agent transmission and spread in susceptible animal populations followed by the quantitative and qualitative process assessment [15].

A quick overview of PPR susceptible wild ruminants. West Caucasian tur (*Capra caucasica*) and Daghestan tur (*Capra cylindricornis*) – cloven hoofed mammals belonging to the *Capra* genus, *Bovidae* family, inhabiting Caucasian mountains at an altitude of 3,000 m above sea level. In winter the animals descend 1,500–2,000 m and cover up to 10 km within the habitat. In summer they are active usually at night and in winter during the day.

Siberian ibex (*Capra sibirica*) – cloven hoofed mammals belonging to the *Capra* genus, *Bovidae* family, inhabiting mountainous regions in Tajikistan, Kyrgyzstan, Mongolia, RF, Kazakhstan, China, Afghanistan, and Pakistan. They are mostly active during the day. They are sedentary animals migrating short distances searching for food. They live at the altitude 500–5,000 m above sea level.

Common ibex (*Capra aegagrus*) – cloven hoofed mammals belonging to the *Capra* genus, *Bovidae* family, inhabiting Turkey, most part of the Asia Minor, in the Caucasus and in a part of Pakistan. The altitudinal range – from 1,200 to 4,500 m above sea level. They are active mostly during the day and travel the distance of 1–2 km per day within the habitat.

Markhor (*Capra falconeri*) – cloven hoofed mammals belonging to the *Capra* genus, *Bovidae* family, inhabiting Western Himalayas, Kashmir, Little Tibet and Afghanistan,

Table 1
Specification of the estimates (scores) for determination of the probability of the disease spread with wild fauna

Factors	Score	Characteristics
a) Inhabiting common territory	1	The habitats of different populations exist separately. The representatives of the population belonging to a certain species don't enter the populations of other species (due to natural or artificial barriers: mountains, territories of other countries, water reservoirs, etc.)
	2	The habitats of different populations exist separately or come into contact with each other. The representatives of the population belonging to one species can enter the population of another species (for instance, populations located in the territory of one country, but in the opposite parts of the country, or hoofed steppe animals, inhabiting the regions at the foot of the mountains where mountain hoofed species live)
	3	The population of one species is a part of the population of another species
b) PPR outbreaks in wild fauna	1	No PPR outbreaks were observed in wild fauna
	2	PPR outbreaks in the wild fauna were observed more than 24 months ago
	3	PPR outbreaks in the wild fauna were observed less than 24 months ago [15]
c) Diurnal activity	1	The periods of grazing and rest don't coincide (for instance, the animals of one population are active during the day and the animals of another population – at night)
	2	Coincidence of the beginning of the rest period of one animal species and the beginning of the active period of another species
	3	Grazing and rest periods coincide

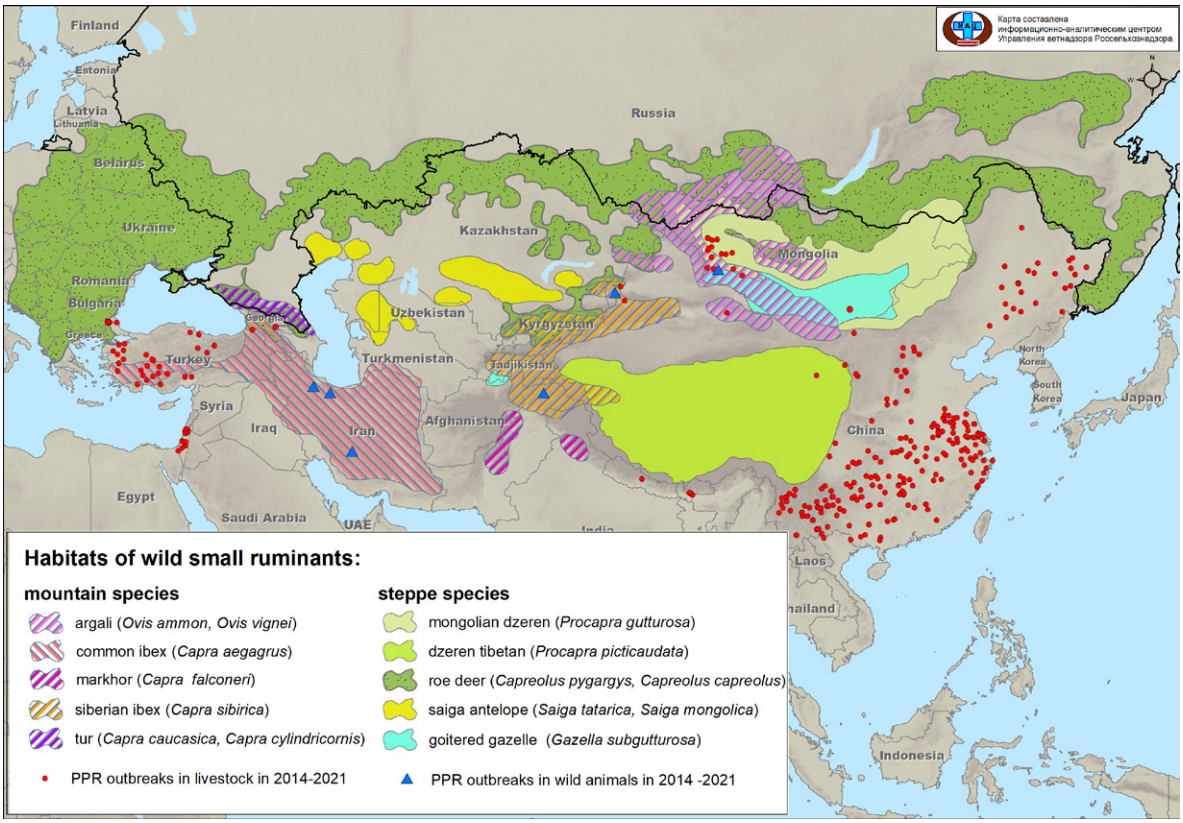

Fig. 1. Habitats of wild small ruminants

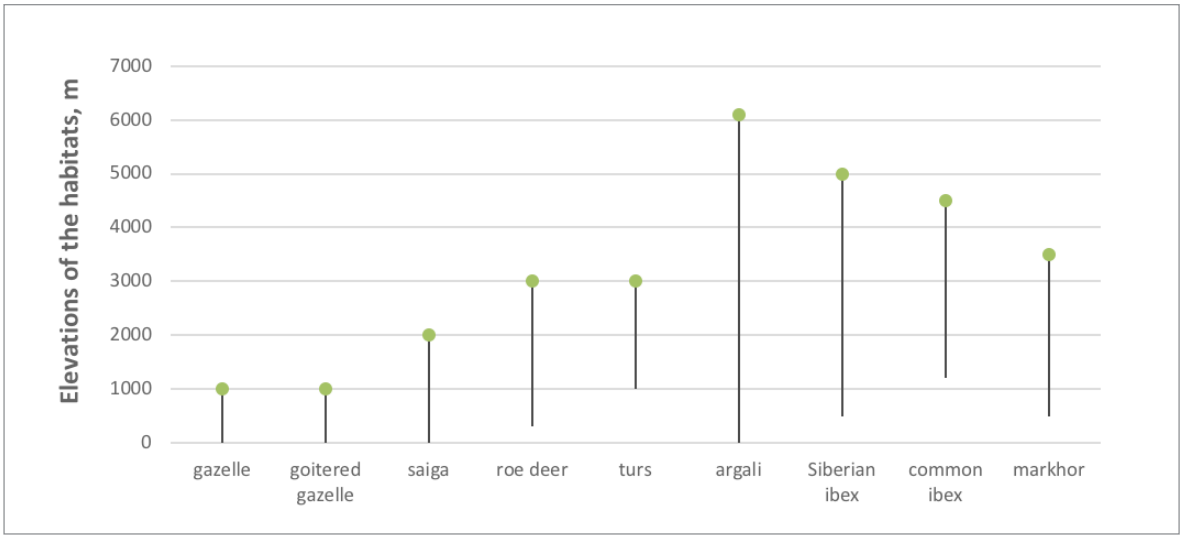

Fig. 2. Distribution of elevations of the habitats of the PPR-susceptible wild ruminants inhabiting the disease infected countries

as well as the mountains along the Panj River, in the Kugitangtau, Babatag and Darvaz ranges in Tajikistan. The altitudinal range is from 500 to 3,500 m above sea level. They are active mainly during the day and cover up to 1–2 km within the habitat per day.

Mountain sheep, or argali, (*Ovis ammon*) – cloven-hoofed mammals from the *Ovis* genus of the *Bovidae* family. Inhabit the mountains and foothills of the West, Central Asia, to the north – to the Transcaucasia, the Kazakh highlands and Southern Siberia, to the south – to the Himalayas, to the east to the Greater Khingan Range, inclusive. They live at an altitude of 1,300–6,100 m above sea level. They are mostly sedentary animals, which can migrate horizontally no more than 5 km. They are active throughout the day resting or grazing.

Mongolian gazelle (*Procapra gutturosa*) and Tibetan gazelle (*Procapra picticaudata*) – cloven hoofed mammals from genus of *Procapra*, *Bovidae* family, inhabiting the

Table 2
Matrix of estimated factors scores of PPR spread with wild ruminants

$X_{a, b, c}$ 1…3*	Turs (*Capra caucasica, Capra cylindricornis*)			Siberian ibex (*Capra sibirica*)			Mountain sheep (*Ovis ammon*)			Common ibex (*Capra aegagrus*)			Markhor (*Capra falconeri*)		
Mongolian gazelle (*Procapra gutturosa*)	1	1	1	2	2	2	3	2	2	1	2	2	1	1	2
	1	2	1	2	2	2	3	2	2	1	2	2	1	2	2
Tibetan gazelle (*Procapra picticaudata*)	1	1	1	1	2	2	1	2	2	1	2	2	1	1	2
	1	1	1	1	1	2	1	1	2	1	1	2	1	1	2
Saiga (*Saiga tatarica*)	1	1	3	1	2	3	1	2	3	1	2	3	1	1	3
	1	1	3	1	1	3	1	1	3	1	1	3	1	1	3
Roe deer (*Capreolus*)	3	1	2	3	2	1	3	2	2	2	2	1	1	1	1
	3	1	2	3	1	1	3	1	2	2	1	1	1	1	1

*

$X_{a, b, c}$ 1…3	Turs (*Capra caucasica, Capra cylindricornis*)		
Mongolian gazelle (*Procapra gutturosa*)	1	1	1
	1	2	1

– parameters a, b, c for turs;

– parameters a, b, c for dzerens.

bunchgrass and partially desert steppes of Central Asia, located at altitudes from 800 to 1,000 m above sea level (Southern Altai, southern Tuva and Transbaikal, Western China, Mongolia, Tibet, Himalayas). Daily activity is polyphasic. They are most active in the evening and at the beginning of the night, as well as in the morning until 10–11 o'clock. In June – July, gazelles feed during the day with short rest breaks. The animals rest most of the night.

Goitered gazelle (*Gazella subgutturosa*) – clove-hoofed mammals from the *Gazella* genus of the *Bovidae* family, living in the desert and semi-desert regions of Tajikistan and Mongolia (the historical habitat included Kyrgyzstan, Iran, Armenia, Georgia and Dagestan). At the beginning of the XXI century, their population encountered 140 thousand animals in total, but by 2009 there was a massive extinction of these ungulates. The average density in the current habitats ranges from 0.003 (Mongolia) to 0.3 individuals per 1,000 ha (Tajikistan). In summer, animals are active from early to late morning. They mix with the Mongolian gazelle population. The total number at the moment does not exceed 11 thousand animals.

Saiga (*Saiga tatarica*) – are migratory bovids. Most of the animals inhabit the southern territories of Kazakhstan, wildlife parks of the Astrakhan Oblast, and the Republic of Kalmykia in the Russian Federation, in Western Mongolia. They live on flatlands, as well as at altitudes reaching 2,000 m above sea level. Animals make seasonal migrations: they spend the winter in places where the height of the snow cover does not exceed 15–20 cm, at the beginning of summer they migrate to more northern regions. In summer, they are active mainly at night, in winter – during the day.

Roe deer (*Capreolus pygargus, Capreolus capreolus*) are New World deer of relatively small size, slender and

Table 3
PPR spread risk levels according to factor scores of PPR spread with wild ruminants

∑X (R)	Turs (*Capra caucasica, Capra cylindricornis*)	Siberian ibex (*Capra sibirica*)	Mountain sheep (*Ovis ammon*)	Common ibex (*Capra aegagrus*)	Markhor (*Capra falconeri*)
Mongolian gazelle (*Procapra gutturosa*)	1.16 (0.38)***	2.0 (0.66)**	2.3 (0.77)*	1.6 (0.55)**	1.5 (0.5)***
Tibetan gazelle (*Procapra picticaudata*)	1.0 (0.33)****	1.5 (0.5)***	1.5 (0.5)***	1.5 (0.5)***	1.3 (0.43)***
Saiga (*Saiga tatarica*)	1.6 (0.55)**	1.83 (0.61)**	1.83 (0.61)**	1.83 (0.61)**	1.6 (0.55)**
Roe deer (*Capreolus*)	2.0 (0.66)*	1.83 (0.61)**	2.16 (0.72)*	1.5 (0.5)***	1.0 (0.33)****

Risk levels: * high risk, ** moderate risk, *** low risk, **** insignificant risk.

Table 4
Average score of PPR introduction risk event

No.	Directions of the introduction	Average score of the possible factors of PPR introduction to the RF ($\bar{X}i, j$)			
		1*	2**	3***	Y*i*
1	Georgia	**4.4**	2.2	4.3	**0.73**
2	Turkey	2.3	1.0	3.9	0.48
3	Iran	2.1	1.1	3.6	0.45
4	Kazakhstan	**4.7**	2.5	4.2	**0.76**
5	China	4.3	2.3	3.6	0.68
6	Mongolia	**4.9**	2.9	**4.4**	**0.81**

* introduction during wild animal migrations;
** introduction with wild animals during mating period;
*** introduction due to contacts between the wild and domestic animals on shared pastures;
Y*i* – PPR introduction *p*-value.

subtle constitution. They exhibit territoriality during the reproductive period. They are sedentary and seasonally migrating populations. Animals live in the forest-steppe, inhabiting semi-deserts and deserts, in high-mountainous regions they ascend to an altitude of 3,000 m above sea level; they are active early in the morning and at dusk. They are widely spread in the southern border regions of the Russian Federation, as well as in adjacent territories. They are kept mostly in wildlife sanctuaries and national parks [17, 19, 20].

Figure 1 shows the cartography of the habitats of wild small ruminants and the diagram of distribution of elevations of the habitats of each described species is shown in Figure 2.

Assessment of PPR introduction with susceptible wild animals. Estimates of factors of PPR spread in the populations of wild susceptible ruminants (Table 2) and the results of their processing (Table 3) were converted to probability values for the potential PPR spread by different populations of wild ruminants (in case there

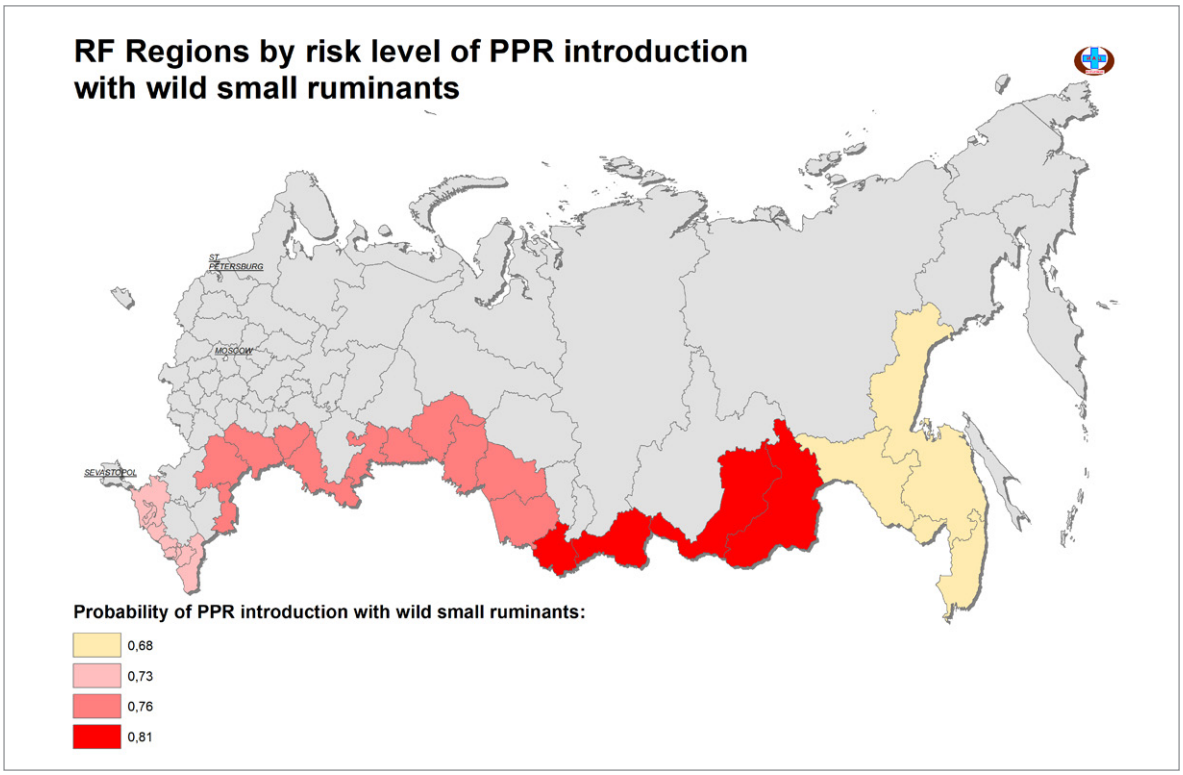

Fig. 3. Russian Federation entities with different levels of PPR introduction risk

are contacts between mountainous and steppe populations).

Based on the data on the behavior of tested animals, their geographical distribution, presence of mountains in the border regions of the Russian Federation and publications, describing the epidemic process in other animals [4, 13], it was suggested that contacts between steppe (migrating) and mountainous (non-migrating) PPR susceptible hoofed animals can play a significant role in the disease spread.

The research showed that interrelations between the Mongolian gazelle (*Procapra gutturosa*) and the mountain sheep (*Ovis ammon*) populations have the greatest potential demonstrating a high risk of the event occurrence (0.77 probability). The animals of these populations inhabit the common area (Mongolia and the northern China), the periods of their activity coincide and PPR outbreaks were recorded in these populations. Considering the fact that these populations are neighbors PPR introduction to the RF is possible in case of outbreaks in these populations.

The contacts between the mountain sheep (*Ovis ammon*) and roe deer (*Capreolus*) also have a high potential for PPR spread (0.72 probability) which indicates that there is a threat of PPR introduction to the Russian Federation with ungulates inhabiting Mongolia.

The least potential for PPR spread may demonstrate the contacts between the Tibetan gazelle (*Procapra picticaudata*) and turs (*Capra caucasica, Capra cylindricornis*), as well as roe deer (*Capreolus*) and markhor (*Capra falconeri*). These animals don't demonstrate any coincidence of the factors, their populations are disconnected and the probability of their inhabiting a common area approaches zero.

Therefore, we can conclude that there is a high risk of PPR spread by the populations of wild susceptible animals inhabiting Mongolia (0.77 and 0.72 probability). It means that it is necessary to enhance the measures preventing the disease introduction in the Far East direction and further disease spread [21].

The results of the expert survey of the specialists in the field of epizootology and small ruminant diseases are given in Table 4 and RF Subject rating according to the risk of PPR introduction to their territory – in Figure 3.

The analysis of the expert survey (Table 4) showed that the most probable factor of the infection introduction to the RF is migrations of potentially infected wild ruminants. Herewith, Mongolia (introduction probability – 0.81), Kazakhstan (introduction probability – 0.76) and Georgia (introduction probability – 0.73) demonstrate high risk. Other directions (China, Turkey and Iran) demonstrate a moderate risk (probability of introduction 0.68; 0.48 and 0.45 accordingly).

CONCLUSION

Based on the work performed a conclusion can be made that PPR outbreaks in wild fauna pose serious threat for the infection spread to the disease free herds. The most probable factor of the disease introduction, according to the experts, is seasonal migrations of wild animals. Presence of many wild animal populations in the Far East direction may contribute to the disease spread. Herewith, the experts consider that the probability of the disease introduction from Mongolia is high (0.81 probability).

When planning activities for preventing PPR introduction and spread in the RF in the framework of activities for maintaining the free status it is important to take into account the results of the research and enhance the surveillance in border regions and in the wild ruminant populations.

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Techniques of blood sampling for detection of African swine fever virus in wild boar and domestic pigs in the field conditions

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SUMMARY

It is thought that due to the high virulence of the African swine fever virus its circulation in the Russian Federation is accompanied by a low seroprevalence. However taking into account a long-term ASF unfavourable situation, the introduction of the virus into the wild boar population, and the occurrence of attenuated viral variants, the significance of serological testing aimed at the detection of viral antibodies is increasing. To collect field samples of biological material from animals for molecular genetic, virological, and serological tests, filter paper, as well as swabs, can be used. The specificity and sensitivity of enzyme-linked immunosorbent assay when testing blood absorbed by filter paper are worse than those shown when testing sera, but they allow effective detection of African swine fever virus antibodies. It was demonstrated that blood absorbed on filter paper can be used for the immunoblot analysis, but the optimum performance could be achieved when the immunoperoxidase technique in combination with samples, taken by swabs was used. When comparing results of enzyme-linked immunosorbent assay performed on sera collected from domestic pigs (infected with ASFV isolates Antonovo 07/14 and Sobinka 07/15), and blood from ear veins absorbed on filter paper the sensitivity was 88.9%, specificity – 90.6%. However, the use of the immunoperoxidase technique for testing blood from swabs showed 100% coincidence with ELISA, while testing of sera with immunoperoxidase technique was superior to ELISA in sensitivity. This means blood sampling using swabs may be recommended for tests after proper validation. This technique can be especially useful for collecting data about infected wild boars because effective eradication strategies are impossible without such data.

Keywords: African swine fever, serological diagnosis, enzyme-linked immunosorbent assay, immunoperoxidase technique, immunoblot analysis, filter paper, swabs

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Методы отбора крови для выявления антител к вирусу африканской чумы свиней у диких кабанов и домашних свиней в полевых условиях

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РЕЗЮМЕ

Считается, что вследствие высокой вирулентности вируса африканской чумы свиней его циркуляция на территории Российской Федерации сопровождается низкой серопревалентностью. Однако ввиду длительного неблагополучия, внедрения в популяцию дикого кабана и появления ослабленных вариантов вируса повышается значимость серологических исследований, направленных на выявление антител к возбудителю болезни. Для сбора полевых образцов биологического материала от животных с целью проведения молекулярно-генетических, вирусологических и серологических исследований можно использовать фильтровальную бумагу, а также зонд-тампоны. Специфичность и чувствительность твердофазного иммуноферментного анализа при исследовании образцов крови, нанесенной на фильтровальную бумагу, уступают результатам, получаемым при исследовании сыворотки крови, но тем не менее позволяют с успехом выявлять специфические антитела к вирусу африканской чумы свиней. Показана возможность использования фильтровальной бумаги, пропитанной кровью животных, для исследования методом иммуноблоттинга, однако оптимального результата удалось добиться при использовании иммунопероксидазного метода в сочетании с пробами, отобранными зонд-тампонами. При сравнении результатов твердофазного иммуноферментного анализа сывороток крови, полученных от домашних свиней (зараженных вирусом африканской чумы свиней изолятов Антоново 07/14 и Собинка 07/15), и крови, отобранной на фильтровальную бумагу при скарификации ушных вен, чувствительность составила 88,9%, специфичность – 90,6%. Однако использование иммунопероксидазного метода при исследовании образцов высушенной на зонд-тампоне крови показало 100%-е совпадение с иммуноферментным анализом, в то время как при исследовании сыворотки крови иммунопероксидазный метод превзошел иммуноферментный анализ по чувствительности. Следовательно, отбор крови с применением зонд-тампонов может быть рекомендован для проведения исследований после соответствующей валидации. Данный метод может быть особенно полезен для сбора информации об инфицированных диких кабанах, так как ее отсутствие делает невозможным применение эффективных стратегий эрадикации.

Ключевые слова: африканская чума свиней, серологическая диагностика, твердофазный иммуноферментный анализ, иммунопероксидазный метод, иммуноблоттинг, фильтровальная бумага, зонд-тампоны

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INTRODUCTION

African swine fever (ASF) is a contagious septic disease of domestic, including ornamental, pigs and wild boars. The disease can be hyperacute to asymptomatic [1–3]. The global ASF situation continues to deteriorate, threatening global food security. At the moment, more than 50 countries are infected with the disease. Dramatic losses are reported in Asian countries. For example, more than 100 million pigs have already been killed and died in China alone [4].

To prevent ASF spread and eradicate the disease in the population of domestic pigs, strict biological safety measures, stamping out policy, and the suppression of illegal trade in pigs and pig products are required. It is more complicated to take similar measures in the wild boar population due to the necessity to stick to biosafety principles in animal habitats and the tendency of the disease to become endemic if suboptimal eradication strategies are used. At the same time, evaluating the ASF control effectiveness is impossible without a properly functioning system of epizootological surveillance [5].

The presence of the virus in the wild boar population creates a risk of infection for domestic pigs. After the ASF virus introduction into the wild boar population, there is a long-term challenge growing in space and time. In most countries of the European Union, since the infection was detected in wild boar, attempts have been made to contain the infection by reducing the population density and, consequently, the number of contacts between infected and susceptible animals in order to prevent transmission of the virus. However, the results of this strategy at an early stage of epizootics in the infected area were unsatisfactory [6].

It is believed that the so-called human factor associated with the violation of veterinary and sanitary rules by people plays an important role in the spread of ASF [7]. At the same time, I. Iglesias et al. using the spatiotemporal analysis, established that in 2007–2013 in the Russian Federation, wild boars could be a source of infection in the populations of domestic pigs in 32.23% of cases and in wild pigs in 28.77% of cases [8].

The presence of infected wild boars increases the complexity of ASF eradication in areas with a large number of low biosecurity backyards. Wild boars are capable of forceful invasion into farm ecosystems, which involves them in the anthropogenic cycle of ASF transmission and allows them to participate in the spread and persistence of the virus in previously free areas [9]. Various biotechnical measures, such as hunting predators and winter feeding of animals, can lead to an increase in the population of wild boars and create conditions for the rapid spread of the infection [10]. Thus, in the system of ASF epizootological surveillance and in the complex of measures taken to eradicate outbreaks, it is necessary to take into account the presence of a susceptible wild boar population in Russia. In addition, according to the requirements of the World Organization for Animal Health (OIE) Terrestrial Code, it is necessary to regularly monitor the population of wild animals for the purposes of ASF and CSF status recognition.

At the moment, serological diagnostics is less effective for the early identification of infected animals than methods for viral antigen or genetic material detection. Thus, from the moment of registration of the first outbreak in 2007 to the present, only a small number of animals have been found to have antibodies to the ASF virus in the tests of swine serum samples using enzyme-linked immunoassay (ELISA) [11].

However, the possibility is not excluded and there is evidence of the occurrence of attenuated ASFV variants, and infection with them may not lead to the rapid death of pigs. Various studies show that in ASF-endemic areas, mortality decreases and the number of asymptomatic and chronic cases grows up, which in the future may require adjustment of the applied diagnosis and control strategies [12, 13].

In addition, at large pig-breeding establishments, where the so-called "naturally occurring death losses" occur, a mild or asymptomatic course of infection may remain unnoticed for up to several weeks [12]. This explains the importance of diagnostic tests aimed at detecting antibodies to the ASF virus, which should help reduce the risk of spreading the disease.

Therefore, in order to determine the real ASF situation, a fast, effective and reliable laboratory diagnosis is necessary. At the same time, in addition to the viral genome identification by polymerase chain reaction (PCR), it is necessary to conduct serological tests for antibodies. It was found that if at the beginning of the epizootic, a greater number of ASF seropositive animals are identified among young animals, then by the late phases of the epizootic process, seroprevalence is higher in animals of older age groups. This fact is probably related to the presence of infected, not convalescent boars [14].

It is believed that due to the high virulence of the ASFV, its circulation in the Russian Federation is associated with low seroprevalence [15]. Nevertheless, serological tests can contribute to the early detection of the disease in wild boar and increase the effectiveness of diagnostic tests used in parallel with virological (molecular biological) diagnostic methods, which can reduce the potentially dramatic consequences of ASF spread [5].

These methods require an appropriate infrastructure and technology for the rapid detection of seropositive wild boars [16]. One of the possible solutions may be the use of immunochromatographic methods [15, 17]. However, they are expensive for large-scale screening tests and are not always applicable [18]. An alternative solution is to detect antibodies in the tissues of internal organs (spleen, lungs, liver) or in the saliva of infected animals [19]. Of all the sample pools tested by researchers in 2008–2012, the percentage of antibody detections in pig tissues using indirect immunofluorescence assay was 45% [20]. However, the concentration of antibodies in such samples, as a rule, is not comparable with their concentration in serum.

By itself, the technology currently used for sampling serum into test tubes with a coagulation activator has shortcomings. Thus, hemolysis is a big challenge, since it is difficult to comply with the technology of sample preparation and transportation in the field, which can lead to false positive results during laboratory diagnostics.

There are a large number of disposable consumables for blood collection available on the market, containing components that accelerate coagulation and facilitate the process of separating serum from a clot. However, the instructions for use do not always provide the necessary information about the composition and properties of all additives. As a result, there may be errors in the results of immunoassay when using blood sampling kits, mainly when testing fragile analytes (cytokines, etc.), which do not include antibodies. On the other hand, some additives are able to interact with antibodies and induce their conformational changes regardless of the type of the solid phase used [21], which can lead to a decrease in the immunospecific activity of antibodies [22].

In the Russian Federation, it is not uncommon for hunters not to send biological material from shot wild boars for testing or not to report detected fallen animals, despite the fact that this is prescribed by the current veterinary regulations [23]. A simple explanation for this is the lack of consumables and the difficulty to take blood samples from dead animals. In order to expand ASF surveillance in the wild, to increase the probability of detecting infected animals and increase the sampling coverage, a simple and inexpensive method of blood sampling on filter paper (FP) can be used. The simplicity of sampling and transportation of samples using FP can help hunters in solving logistical problems of delivering samples to the laboratory [24]. Blood samples can be taken without any special equipment and special training, including from recently shot animals. This approach will allow agent identification and antibody testing, using a single sample easily obtained in the field [25].

At the same time, swabs are successfully used for virological studies, which is especially important when carcasses of wild boars are found, since their destruction is an important link in anti-epizootic measures, and the natural decomposition of the remains can take from several days in the summer to several years under appropriate conditions [26].

The use of FP samples and swabs for serological studies. The method of drying adsorbed blood on FP with subsequent elution and testing of various analytes has been successfully used in diagnostic tests since 1927 [27].

Currently, FP is an affordable option for collecting biological samples to diagnose various infectious diseases by serological and molecular biological methods. At the same time, the sensitivity and specificity of solid-phase enzyme-linked immunoabsorbent assay (SP-ELISA) when using FP decrease compared to the use of blood serum, although in most cases it remains at an acceptably high level [25, 28–30]. However, reduced sensitivity is a potential limitation of FP samples in protocols that require undiluted serum [28].

Modern FP can be divided into two main types. The first is carriers specially designed for the storage and isolation of nucleic acids. This type of FP is impregnated with substances that lyse cells, denature proteins, inactivate biological material and protect nucleic acids from the nuclease action. Carriers of the second type do not contain an inactivation component and can be used for virus isolation and serological studies [31].

When comparing the effectiveness of antibody detection by the ELISA method for blood samples adsorbed on FP and SP-ELISA serum samples obtained from pigs, the results of the studies showed comparable sensitivity [31]. It has also been shown that the ASF virus is isolated from the blood-soaked FP after 9 months of storage at 37 °C on a sensitive cell culture and can be genotyped. Nevertheless, the use of FP for the detection of genetic material goes along with the decrease in the sensitivity [32].

Blood sera can be stored frozen for a long time. However, their subsequent testing shows a progressive decrease in the number of detected antibodies by most of the diagnostic methods used [28]. Various studies have shown that samples adsorbed on FP can be stored for a relatively long period with a slight decrease in the titer due to antibody destruction. Using immunoblotting, it was found that a decrease in the titer of antibodies is observed in all detectable protein epitopes [33].

Temperature and humidity play an important role in the degradation of the analyte [29]. For example, the antibody titer to the human immunodeficiency virus decreases by approximately 15% during 30 days of storage at room temperature and uncontrolled humidity. At higher temperatures, titer loss increases. Samples stored under controlled humidity conditions (with a dissectant) at room temperature remain stable up to 190 days. At low temperature and humidity, antibodies to the human immunodeficiency virus on FP are stable for at least 56 months [33].

In the experiments of P. S. Curry et al. when comparing samples on FP after one year, compared to frozen serum stored for the same period, sensitivity was > 88% (for all but two assays), and specificity remained > 90% [28].

General recommendations for increasing the storage period is the need for complete drying of the blood applied to the FP, storage in a place protected from direct sunlight and at a humidity of no more than 30%. For short-term storage (up to two years) at a temperature of 4 °C it is recommended to use re-sealable zipper storage bags (ziplocks) with a dissector and a humidity indicator. Long-term storage is recommended at minus 70 °C [28, 33].

Thus, an important link in the diagnosis of various infectious diseases is the observance of temperature conditions during the transportation of samples to the laboratory and their storage. For example, according to the World Health Organization, more than 50% of the vaccines produced in the world are lost. At the same time, vaccine losses in unopened vials are usually associated with cold chain and logistics problems [34]. Twenty-five percent of the vaccines produced in the world are delivered to the destination in various stages of degradation due to violation of conditions during delivery [35]. Moreover, according to the Global Alliance for Vaccines and Immunization, half of the health care institutions in the poorest countries do not have access to electricity at all, and only 10% have uninterrupted power supplies [36].

At the same time, the practice of using swabs by many laboratories for sampling and testing by various methods (PCR, commercial ELISA kits) demonstrates the versatility of the samples obtained, as well as the simplicity of their adaptation to the conditions of a particular laboratory.

Therefore, swabs and FP are a good option for serum sampling to perform tests for ASF. They have a number of advantages: the samples adsorbed on swabs or FP are easy to handle, can be stored for long periods, can be separated if repeated or additional diagnostic tests are required. Thus, it is a practical, inexpensive and simple approach for ASF epizootological surveillance in wild boar [30].

The aim of the work was to experimentally confirm the possibility of using filter paper and swabs in sampling for serological testing aimed at detecting ASFV specific antibodies, as well as comparing various methods of testing and sampling.

MATERIALS AND METHODS

The following materials were used for studies and experiments:

– domestic pigs (crossbreeds of large white, landrace, duroc breeds), delivered from a farm, free from major infectious diseases of pigs of the Vladimir Oblast;

– wild European wild boars (8 animals, age 3–4 months), imported from the ASF free farm of the Kostroma Oblast;

– ASFV isolates: Shikhobalovo 10/13, isolated from a dead wild boar in the territory of the Yuryev-Polsky Raion of the Vladimir Oblast; Antonovo 07/14, isolated from a domestic pig in the village of Lobok, Nevelsky Raion, Pskov Oblast; Sobinka 07/15, isolated from a wild boar in the hunting farm of the Vladimir Oblast; low virulent ASF virus of the 60th passage, obtained in cell culture CV-1 – ASF/ARRIAH/CV-1/60;

– Class 3 filter paper (Whatman®, UK);

– swab (cotton swab) with a viscose tip (Ningbo Greetmede Medical Instruments Co., Ltd, China).

All animal experiments were carried out in strict accordance with the interstate standards for the care and use of laboratory animals adopted by the Interstate Council for Standardization, Metrology and Certification, as well as in accordance with the requirements of Directive 2010/63/EU of the European Parliament and of the Council of the European Union of 22.09.2010 on the protection of animals used for scientific purposes.

Enzyme-linked immunosorbent assay was performed using the INgezim PPA Compac kit (Ingenasa, Spain) in accordance with the kit instructions.

Immunoblotting was performed using commercial reagents (CISAINIA, Spain) in accordance with the manufacturer's instructions.

The immunoperoxidase technique (IPT) for detecting antibodies to the ASF virus was carried out in accordance with the guidelines of the FGBI "ARRIAH"[1].

RESULTS AND DISCUSSION

During the bioassay on wild boars and domestic pigs infected with the ASFV Shikhobalovo 10/13 isolate [3], the possibility of using FP for blood sampling from dead wild boars and subsequent testing for antibodies was evaluated. For this purpose, a few hours after the death of the animals the filter paper was soaked in the unclotted blood flowing out the dissected vessels in the process of the partial evisceration during the autopsy, mainly in the thoracic cavity. Since systemic biochemical processes take place in the blood after the death of the animal, blood must be sampled as soon as possible. It is advisable to use whole blood, since, despite the fact that the serosanguinous fluid contains antibodies, it is not equivalent to whole blood [28]. Paired blood samples were taken from domestic pigs into test tubes with a coagulation activator and on FP.

The filter paper was dried at room temperature in the animal facilities and placed in separate re-sealable bags. About 0.5 cm^3 of blood was required to impregnate 1 cm^2 of FP. ELISA was performed 3 days after sampling. The ASF ID Screen® African Swine Fever Indirect (IDvet, France) kit for the detection of ASFV antibodies is commercially available, which has a protocol for testing blood filter paper samples [24]. There are also specially manufactured versions of commercial kits for testing FP samples [31]. However, in this experiment, the possibility of using the Ingezim PPA Compac kit (Ingenasa, Spain) followed by immunoblotting confirmation was tested.

During immunoblotting testing of a sample taken from a wild boar, the several slightly colored stripes were observed on the nitrocellulose membrane, which indicates the formation of an immune complex in areas containing ASF virus proteins and, consequently, the presence of antibodies in the sample. Stripes are not formed when testing negative FP samples. When comparing samples obtained from a hyperimmune piglet by the conventional method and using FP, it is seen that the intensity of staining decreases in all areas of the nitrocellulose membrane coated with a specific antigen. However, the samples remain positive.

Various methods of using FP were studied. In the first case, the paper was placed in the sample buffer in a volume of 5 cm^3 on 5 cm^2 of the FP area, which is comparable to the ratio prescribed in the instructions for the serum kit, and was kept at room temperature for 5 minutes for elution. The resulting eluate was introduced in a volume of 0.1 cm^3 per well as test samples.

In the second case, the FP placed in the sample buffer was kept for 3 minutes in a homogenizer with active stirring. Before being introduced into the wells of the microplate, the eluate was centrifuged to precipitate the formed blood elements and FP particles for 3 minutes at 1000 g.

In the third case, circles with a diameter of about 3 mm were cut out of the dry FP and placed in the well of a microplate with a buffer added. According to V. G. Pomelova et al., within one hour, more than 90% of antibodies are extracted from a disk with a 3.2 mm diameter placed in the well of a coated microplate [25]. However, taking into account the average FP impregnation capacity, the blood:buffer ratio was approximately 1:30, which is lower than the serum working dilution of the kit used (1:2).

For a reliable comparison of test methods for samples obtained by different methods, it is necessary to compare paired samples of blood applied to the FP and blood serum taken from live animals. However, in our experiment, due to the high viral virulence, which leads to rapid death, the animals did not have time to develop antibodies. In blood samples taken 2 or 3 days before death (6–12 days after infection, according to the sampling schedule – once every 3 days), antibodies were not detected in serum. Therefore, the blood applied on the FP during autopsy was tested using SP-ELISA and compared with the results of immunoblotting, since this method is highly specific and highly sensitive.

When comparing the SP-ELISA results of sera obtained from domestic pigs (infected with ASFV isolates Antonovo 07/14 and Sobinka 07/15), and blood sampled on FP during scarification of the ear veins, sensitivity was 88.9% (with a 95% confidence interval – from 65.3 to 98.6%), specificity – 90.6% (79.3–96.9%).

The sensitivity of the method using FP circles cut out by the well diameter, placed directly into the well of a microplate with a buffer, was low when tested by this kit. Probably, this method can be recommended only for use in kits that allow high dilution of samples or using staining enhancers. The method without centrifugation showed a large number of false positive results. The method with homogenization and centrifugation of the sample showed the best sensitivity and specificity (Table 1).

When studying the results presented in Table 1, it can be seen that the use of FP leads to both false positive and false negative results. When comparing the data obtained by testing of FP blood samples using immunoblot analysis and SP-ELISA, it was found that one negative sample also showed a false positive result and SP-ELISA.

The results obtained in this experiment are consistent with the data published by T. Randriamparany et al. [31], who tested ASF FP blood samples. In the mentioned work, slight decrease in sensitivity was also observed. At the same time, the authors concluded that the method of blood sampling using FP is a suitable method for collecting and storing samples.

In the experiments conducted by J. Carlson et al., testing blood swab samples by commercial ELISA kit when compared to serum samples showed sensitivity of 93.1% (95% confidence interval, 83.3–98.1%), and 100% specificity (95.9–100.0%). The authors, therefore, concluded, that the swabs are suitable for sampling and subsequent testing for ASFV antibodies by ELISA. In addition, field tests of swabs during sampling from decomposed carcasses of wild boars in an endemic area of Estonia by PCR also showed high accuracy of the results [26].

[1] Metodicheskie rekomendatsii po vyyavleniyu antitel k virusu afrikanskoi chumy svinei immunoperoksidaznym metodom = Methodological recommendations for the detection of antibodies to the African swine fever virus by the immunoperoxidase method: approved by FGBI "ARRIAH" on 19.11.2020 No. 68-20. Vladimir: FGBI "ARRIAH"; 2019. 12 p. (in Russ.)

Table 1
Comparison of sensitivity and specificity of SP-ELISA and immunoblot analysis when testing sera and blood absorbed on filter paper

SP-ELISA				Immunoblotting		
Filter paper blood sample	Serum					
	Pos.	Neg.	Total	Pos.	Neg.	Total
Pos.	16	5	21	3	1	4
Neg.	2	48	50	0	4	4
Total	18	53	71	3	5	8

pos. – positive result;
neg. – negative result.

In the current conditions, when there is practically no testing for ASFV antibodies performed in wild boar samples, the use of methods that simplify the sampling procedure from wild animals, even taking into account some decrease in sensitivity, will enable to collect valuable information about the dynamics of ASF spread. Therefore, the possibility of detecting antibodies using a more sensitive immunoperoxidase technique (IPT) was tested.

To compare the methods of blood sampling, IPT and ELISA were used. The samples were represented by blood collected by ear vein scarification using swabs (Fig. 1), as well as serum taken from domestic pigs on the 10th and 31st days after infection with ASF virus (ASF isolate/ARRIAH/CV-1/60). The results of the tests are presented in Table 2.

Thus, the use of IPT for testing dried blood swab samples (Fig. 2) showed a 100% match with ELISA, while IPT used for testing serum even surpassed the sensitivity of ELISA. Therefore, blood sampling using swabs can be recommended for testing after appropriate validation. This method will be especially useful for collecting information about infected wild boars, since the lack of data makes it impossible to use effective eradication strategies.

Wild boar play an important role in the ASFV spread. Disease surveillance can be based both on testing of dead boar carcasses (passive surveillance) and on the detection of virus or antibodies in shot/caught wild boars (active surveillance). Wild boars can be a reservoir for the infection, regardless of the virus circulation in the population of domestic pigs. At the same time, passive surveillance provides a higher probability of early detection of ASF, especially during the first year in the early stages of the epizootics.

The use of serological testing methods has a low diagnostic value at the initial stages of the epizootics. Nevertheless, active surveillance aimed at the identification of seropositive wild boars may be preferable at later stages (in the endemic phase) of the epizootics [5].

Consequently, in order to increase the effectiveness of ASF epizootological surveillance in wild boar, especially at

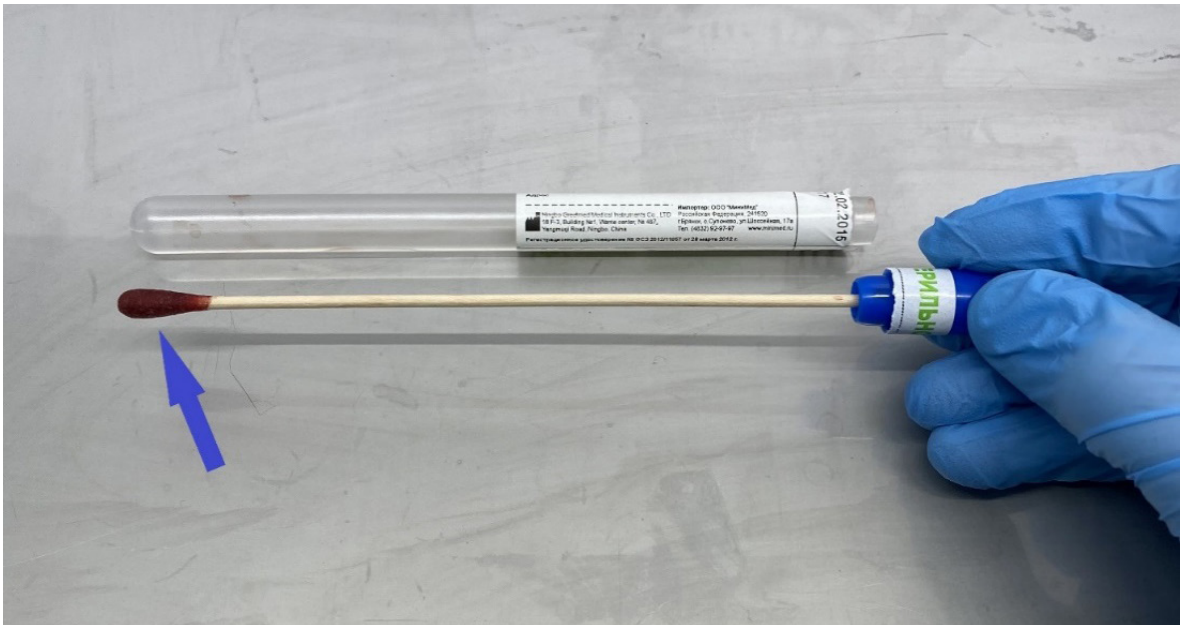

Fig. 1. Blood sample absorbed on the swab

Table 2
Comparison of results of serum and blood swab testing by ELISA and immunoperoxidase technique

Days after infection	ELISA (serum)	IPT (swabs)	IPT (serum)
10	+	1:20	1:320
10	+	1:5160	1:81920
10	–	–	1:20
10	+	1:640	1:640
31	+	1:20	1:320
31	+	1:1280	1:20480
31	+	1:1280	1:10240
31	+	1:80	1:5160
31	+	1:160	1:40960
31	+	1:160	1:81920

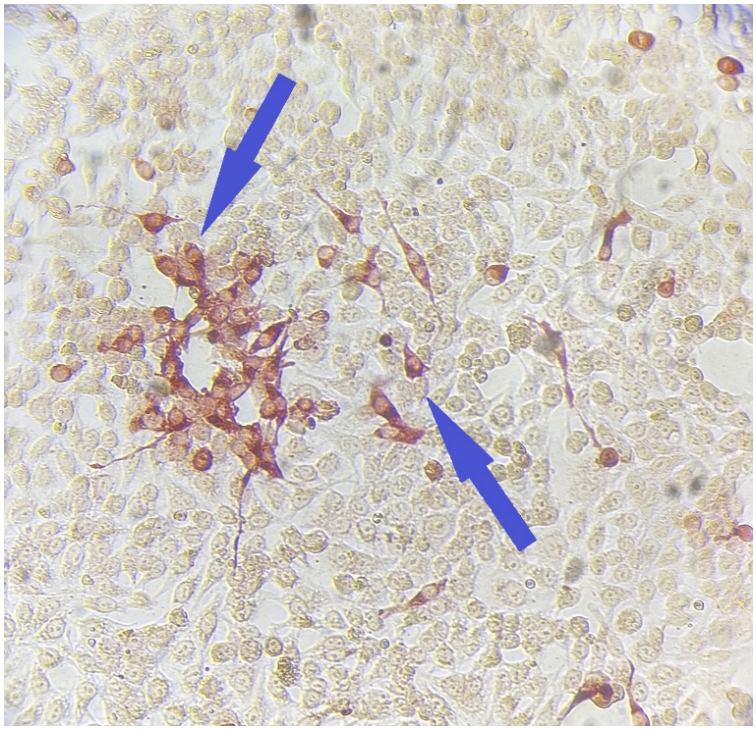

Fig. 2. Stained cytoplasm of infected cells when testing blood samples from swabs (magnification 400×)

the late stages of epizootic, serological tests are necessary, while the use of alternative sampling methods is justified.

CONCLUSION

The use of even the most highly accurate diagnostic methods may be limited by the quality of the delivered samples. And since it is required to detect not only the ASFV antigen or genetic material, but also antibodies to it, an alternative may be blood sampling using FP or swabs. Thus, it is possible to take samples from recently died and shot animals. In domestic pigs, blood samples can be collected by simple scarification of the ear vein without the use of test tubes, which reduces the risk of environmental contamination when they are destroyed or unintentionally opened, since the sample is not fluid. The use of FP as a sample carrier can reduce the amount of space required for long-term storage of the sample in a frozen state. The disadvantages of such sample preparation include a decrease in the sensitivity of the diagnostic methods used, however, the use of FP allows increasing the mass coverage of tests for the main purposes of the surveillance (early detection, prevalence determination,

and evidence of the population freedom). Therefore, the concept of using swabs and IPT is very promising, and in some cases there is no alternative, especially after appropriate validation and, possibly, the selection of swabs that provide more effective adsorption of samples, and can be recommended for the detection of ASFV antibodies in wild boar and domestic pigs. This approach combines the advantages of using FP with the high sensitivity and specificity of this diagnostic method.

The use of FP or swabs is most feasible when conducting mass screenings of target animal populations within the ASF surveillance (including during state monitoring). Blood samples can be collected using this method anywhere by minimally trained personnel, while the cold chain is not required to be maintained.

There are various special types of FP and swabs, therefore, it is necessary to select a material that ensures effective adsorption of the sample, optimal cost of collection, transportation and storage of samples. Before use, calibration will be required for the corresponding method, or a diagnostic kit.

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Comparative effectiveness of eimeriocidal products for treatment of broiler chickens in small scale production

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SUMMARY

There is currently almost no poultry holding where avian eimerioses, both monoinvasions and those associated with cryptosporidioses, salmonelloses and colibacterioses, are not reported. In view of this, the disease control is an urgent challenge that shall be approached in its entirety, using various eimeriostats, antibiotics and probiotics. Searching for new effective products with broad-spectrum antiparasitic action is one of priorities in avian eimeriosis control. Comparative tests of different combinations of eimeriocidal products, namely solicox + chicktonic, maduvet + tylosin and eimeterm + enrofloxacin, for their treatment and protective effectiveness were carried out under production conditions in broiler chickens of a poultry factory located in the Republic of Dagestan. To perform the tests, four groups of broiler chickens (one control group and three test groups, each comprising 50 chickens) were formed based on the principle of analogues. The treatment and prevention scheme adopted in the said poultry holding was used for the control group chickens. Test group 1 chickens were given solicox at a dose of 2 ml per 1 liter of drinking water in combination with chicktonic (a feed supplement) at a dose of 1 ml per 1 liter of water during 4–5 days. Group 2 chickens were given maduvet at a dose of 3–5 mg/kg of body weight with feed twice and tylosin at a dose of 5 g of powder per 10 liters of water once a day during 5 days; where necessary, the procedure was repeated in 14–16 days. Group 3 broiler chickens were given eimeterm 2.5% at a dose of 7 mg/kg of body weight with water during 2 days in combination with enrofloxacin at a dose of 3 ml per 1 liter of drinking water during 5–6 days. It is shown that a decrease in the number of clinically diseased and dead chickens was observed in the test groups after the use of eimeriocidal products that helped to improve zootechnical parameters of reared broiler chicks. Product extenseffectiveness and intens-effectiveness in different groups of chickens were as follows: Group 1 – 89.2 and 96%, Group 2 – 87.6 and 94%, Group 3 –81.4 and 96%, respectively. The clinical signs of eimeriosis were observed in the control group chickens throughout the entire period of studies, invasion extensity and intensity were 87.6 and 42.6%, respectively.

Keywords: eimeriosis, coccidiostat, solicox, chicktonic, maduvet, tylosin, eimeterm, enrofloxacin, effectiveness, droppings, broiler chickens, live weight, treatment effectiveness, oocyst, caeca

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Сравнительная эффективность эймериоцидных препаратов при лечении цыплят-бройлеров в условиях мелкотоварного производства

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РЕЗЮМЕ

В настоящее время практически нет ни одного птицеводческого хозяйства, где бы не регистрировались эймериозы птиц, как моноинвазии, так и в ассоциации с криптоспоридиозами, сальмонеллезами и колибактериозами. С учетом этого факта борьба с данным заболеванием является актуальной задачей, которую необходимо решать комплексно, с применением различных эймериостатиков, антибиотиков и пробиотиков. Изыскание новых эффективных препаратов, обладающих широким спектром антипаразитарного действия, – одно из приоритетных направлений в борьбе с эймериозами птиц. В производственных условиях одной из птицефабрик Республики Дагестан на цыплятах-бройлерах провели сравнительные испытания различных комбинаций эймериоцидных препаратов: соликокс + чиктоник, мадувет + тилозин и эйметерм + энрофлоксацин – с целью выявления их лечебной и профилактической эффективности. Для проведения исследований по принципу аналогов сформировали четыре группы цыплят-бройлеров: одна – контрольная и три – опытные – по 50 голов в каждой. В контрольной группе использовали схему лечебно-профилактических мероприятий, принятую в данном птицеводческом хозяйстве. Цыплята-бройлеры первой опытной группы получали препарат соликокс в дозе 2 мл на 1 л питьевой воды в сочетании с кормовой добавкой чиктоник в дозе 1 мл на 1 л воды в течение 4–5 дней. Цыплятам второй группы с кормом задавали препарат мадувет в дозе 3–5 мг/кг массы тела двукратно и тилозин в дозе 5 г порошка на 10 л воды один раз в сутки в течение 5 дней, при необходимости процедуру повторяли через 14–16 дней. Цыплята-бройлеры третьей группы два дня получали с водой 2,5%-й препарат эйметерм в дозе 7 мг/кг массы тела в комбинации с энрофлоксацином в дозе 3 мл на 1 л питьевой воды в течение 5–6 дней. Показано, что в опытных группах после применения эймериоцидных препаратов наблюдали уменьшение количества клинически больных особей и снижение падежа, что способствовало улучшению зоотехнических показателей при выращивании цыплят-бройлеров. Экстенс- и интенсэффективность препаратов в первой группе цыплят составила 89,2 и 96%, во второй группе – 87,6 и 94%, в третьей – 81,4 и 96% соответственно. У птиц контрольной группы наблюдали клинические признаки эймериоза в течение всего периода исследования, а показатели экстенсивности и интенсивности инвазии составили 87,6 и 42,6% соответственно.

Ключевые слова: эймериоз, кокцидиостатик, соликокс, чиктоник, мадувет, тилозин, эйметерм, энрофлоксацин, эффективность, помет, цыплята-бройлеры, живая масса, лечебная эффективность, ооциста, слепые отростки

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INTRODUCTION

Eimeriosis is one of the most common diseases in poultry husbandry. The disease is caused by protozoans of the genus *Eimeria*, which propagate in the intestine and cause digestion and absorption disorders, bodily dehydration, gastrointestinal wall bleeding, thereby increasing the chances of infection with other agents. At present, eimeriosis still continues to be a problem. Unfortunately, there are almost no poultry farms where this parasite is not present. When veterinary and sanitary rules are not complied with, large amounts of *Eimeria* can accumulate in poultry houses within a short space of time, thus constituting a continuing threat of invasion occurrence and spread, and cause huge damage. Many domestic and foreign scientists have addressed this issue [1–10].

Like many parasitic infections, eimeriosis mainly affects young poultry, since they are still developing their immunity during the postnatal period. According to many researchers, coccidiosis affects chickens aged from 10 days to 3 months [11, 12].

Infection in poultry usually occurs during warm and humid seasons when conditions are favourable for the growth of exogenous phase *Eimeria* and their persistence in the environment. Such seasons are late autumn, winter and early spring in the southern regions of Russia and late spring, summer and early autumn in the north west of the country.

According to the data from a number of researchers, the disease control is complicated by the fact that chickens are affected by 9 *Eimeria* species having different sensitivity to eimeriostats. A certain *Eimeria* species can infest only one poultry species and is absolutely harmless for another species, i.e. the disease agents are monotropic. S. K. Svanbaev [13] studied morphologically similar *Eimeria* isolated from turkeys and chickens and found them to be nonidentical, no cross-infection occurred.

Poultry that have had eimeriosis caused by one *Eimeria* species remain susceptible to infection caused by another species. Due to the short life cycle and high productivity of *Eimeria*, the proportion of mass outbreaks in the poultry

houses is continuing to increase, and eimeriosis is currently considered to be the most costly disease.

Eimerioses often occur in association with cryptosporidioses, salmonelloses and colibacterioses, thus constituting a serious threat for poultry holdings of various forms of property. Even the mild form of eimeriosis in association with cryptosporidia, along with inadequate feeding and in the presence of other unfavourable factors, inflicts significant economic damage on poultry farming. Global economic losses due to eimeriosis are huge and make more than 3 billion US dollars a year [14, 15]. The damage is made up of expenses associated with chicken deaths, growth retardation, reduced meat production, increased feeding and treatment costs [16]. Therefore, the invasion control methods undergo continuous improvement, and new methods are developed [2, 5–7, 14, 16–27].

The long-term application of the same eimeriostats results in the appearance of resistant forms of coccidia. Many domestic and foreign authors address this problem in their papers [4, 22, 24, 28–32].

In view of this, treatment shall involve the alternating use of various products, provided that the product administration doses and schedules are complied with. At present, the control of associative forms of avian eimerioses is an urgent challenge that shall be approached in its entirety, using various eimeriostats, antibiotics and probiotics. Searching for new highly effective eimeriostats with broad-spectrum antiparasitic action is increasingly important.

The study was aimed at the examination of comparative effectiveness of currently available eimeriocidal products for treatment of broiler chickens.

MATERIALS AND METHODS

The studies were carried out at the Laboratory for the Study of Invasive Diseases of Farm Animals and Poultry of the Caspian Regional Research Veterinary Institute – Branch of Dagestan Agriculture Science Center and at the AO "Poultry factory "Makhachkalinskaya" affected with avian eimerioses.

Comparative tests of different combinations of eimeriocidal products, namely solicox + chicktonic, maduvet + tylosin and eimeterm + enrofloxacin, for their treatment and prevention effectiveness were carried out under production conditions.

To perform the tests, 200 fourteen-day-old Ross-308 broiler chickens were selected based on the principle of analogues; four groups (one control group and three test groups, each comprising 50 chickens) were formed.

The treatment and prevention scheme adopted in the mentioned poultry holding was used for the control group chickens.

Test group 1 chickens were given solicox at a dose of 2 ml per 1 L of drinking water and chicktonic at a dose of 1 ml per 1 L of water during 4–5 days.

Group 2 chickens were given maduvet at a dose of 3–5 mg/kg of body weight with feed twice and tylosin at a dose of 5 g of powder per 10 L of water once a day during 5 days; where necessary, the procedure was repeated in 14–16 days.

Group 3 broiler chickens were given eimeterm 2.5% at a dose of 7 mg/kg of body weight with water (this is equivalent to 1 ml of the product per 1 L of drinking water) during 2 days and enrofloxacin at a dose of 3 ml per 1 L of drinking water during 5–6 days.

Table 1
The scheme of the experiment carried out in Ross-308 broiler chickens

Groups	Product	Number of chickens	Product dose and treatment schedule
test group 1	solicox + chicktonic	50	2 ml/1 L of water + 1 ml/1 L of water during 4–5 days
test group 2	maduvet + tylosin	50	3–5 mg/kg of body weight twice + 5 g/10 L of water once a day during 5 days; where necessary, the procedure should be repeated in 14–16 days
test group 3	eimeterm 2.5% + enrofloxacin	50	7 mg/kg of body weight (which is equivalent to 1 ml of the product per 1 L of drinking water) + 3 ml/1 L of water during 5–6 days
control group	–	50	–

The scheme of the experiment is presented in Table 1.

The experiment in animals was carried out in compliance with GOST 33215-2014 adopted by the Interstate Council for Standardization, Metrology and Certification and according to the requirements of the Declaration of Helsinki (2000) and Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes.

The following material was used for testing: samples of droppings from litter and feedstuffs, floor and tool swabs, as well as caecum samples from dead poultry.

Coprological examination of the chickens' droppings was carried out before the start of the experiment and on days 20, 26, 36, 46 after the product administration.

The intensity of *Eimeria* infestation in poultry was determined according to Darling's method standardized by N. P. Orlov, droppings were tested using direct smears stained with methylene blue, as well as flotation and centrifugation technique (Nikitin and Breza).

Infestation level, the intensity (II) and extensity (EI) of *Eimeria* invasion in the control and test group chickens were determined by counting oocysts in 1 g of droppings using a McMaster counting chamber or the counting chamber developed by the Scientific Research Institute Parasitology named after K. I. Skryabin (VIGIS) in 20 microscope fields of view.

The intenseffectiveness (IE) of the eimeriocidal products was calculated according to the formula:

$$IE = \frac{C - P}{C} \times 100\%,$$

where C is the geometric mean number of oocysts in the control group chickens;

P is the geometric mean number of oocysts in the test group chickens.

The extenseffectiveness (EE) of the products was determined based on the number of broiler chickens that became completely oocyst free after the treatment.

Table 2
The results of comparative studies of product effectiveness and some zootechnical parameters of reared broilers

Parameter	Group			
	control group	test group 1	test group 2	test group 3
Before treatment				
Number of chickens in the group	50	50	50	50
Age of chickens, days	16	16	16	16
Average weight of one chicken at the beginning of the experiment, g	119	121	119	120
Oocysts in caeca, mean number per FOV	42.6 ± 3.2	35.8 ± 3.5	39.4 ± 3.8	37.6 ± 4.2
Oocysts in 20 dropping samples, mean number per FOV	36.8 ± 2.6	31.4 ± 3.8	33.4 ± 3.2	34.7 ± 3.6
After treatment				
Number of chickens that died during the observation period (46 days)	17	2	3	2
Mortality rate, %	34	4	6	4
Oocysts in caeca, mean number per FOV	47.9 ± 5.3	2.4 ± 1.3	6.1 ± 1.1	3.3 ± 0.96
Oocysts in 20 dropping samples, mean number per FOV	44.8 ± 4.2	1.9 ± 2.3	4.8 ± 1.7	2.1 ± 1.3
Product intenseffectiveness, %	–	95	88	94
Survival rate during the observation period (46 days), %	66	96	94	96
Daily average live weight gain during the observation period (46 days), g	36	47	44	46
Average feed intake per 1 kg of live weight gain during the observation period (46 days), kg	2.46	2.1	2.2	2.15
Live weight at the time of slaughter, g	1,725	2,125	1,980	2,050

Number per FOV – number per microscope field of view.

In order to detect *Eimeria* and morphological lesions in the digestive tract, the chickens were selectively subjected to necropsy.

The therapeutic effectiveness of the products was assessed based on the results of coproscopic examination carried out for detection of *Eimeria* in poultry caeca, droppings and swabs from various surfaces of the production facilities.

The following performance indicators were taken into account: survival rate for each poultry house, body weight gain and feed conversion ratio.

The eimeriostat test results were statistically processed using the Biometrics software.

RESULTS AND DISCUSSION

Intensity and extensity do not always characterize the invasion process comprehensively. To get a clear picture of the disease, one should perform a complete helminthological necropsy, taking into account the status of internal organs and the severity of intestinal lesions in poultry.

Table 2 shows the results of comparative studies of the product effectiveness, as well as some zootechnical parameters of reared broilers during the observation period.

The tests showed that the clinical signs of eimeriosis were reported in the control group chickens throughout the entire observation period, and in the test group chickens – only until the treatment started. During the 46 days of the experiment, 17 chickens of the control group died, the survival rate was 66%.

The necropsy of the fallen chickens revealed that the most pronounced lesions were found in caeca: the caecal cavity was filled with blood clots, the mucosa was thickened, necrotic foci were observed in some places. The walls of the duodenum were thickened, petechiae were observed. Such diagnoses as typhlitis and duodenitis were established.

The test group poultry looked clinically healthy. During the entire observation period (46 days), 2, 3 and 2 chickens died in groups 1, 2, 3, respectively, i.e. the survival rates were 96, 94 and 96%. The necropsy of the fallen chickens did not reveal any lesions characteristic of avian eimeriosis in their internal organs and intestines.

Daily average weight gain during the observation period (46 days) in the control group was 36 g, and in the test groups – 47, 44, 46 g, respectively. Feed intake per 1 kg of live weight gain in the control group was 2.46 kg, and in the test groups – 2.1, 2.2 and 2.15 kg, respectively. At the end of treatment, average live weight of one chicken in the control group was 1,725 g, and in the test groups – 2,125, 1,980 and 2,050 g, respectively, i.e. 114–400 g more than in the control group.

During the rearing period, the control group chickens demonstrated slower growth and development and failed to gain the same live weight as the test group chickens. The control group chicken mortality was 5–6 times higher than that of the test group chickens.

Coprological examination revealed the presence of three *Eimeria* species in the chickens' droppings: *E. tenella*, *E. maxima*, *E. acervulina*; the intensity of invasion in the control group was 42.6 ± 3.2 oocysts in 20 microscope fields of view, and in the test groups – 35.8 ± 3.5; 39.4 ± 3.8; 37.6 ± 4.2.

It was found that, after the tested products had been applied, the test group broiler mortality decreased considerably, the number of oocysts in caeca and in 20 dropping samples reduced to 2–6 oocysts in the microscope field of view. The test results showed that the products have pronounced eimeriocidal effect against *Eimeria* oocysts, as well as high therapeutic effectiveness that was 95, 88 and 94% for test groups 1, 2 and 3, respectively, after the treatment.

Data on the results of studies of the extensity (EI) and intensity (II) of *Eimeria* invasion and the extenseffectiveness (EE) and intenseffectiveness (IE) of the studied eimeriocidal products in dynamics are presented in Table 3.

The test results showed that, after the products had been used, the extensity and intensity of invasion on day 46 of testing in group 1 chickens were 9.3 and 1.1%, in group 2 chickens – 6.7 and 2.1%, in group 3 chickens – 7.3 and 1.5%, respectively.

On day 46 of observation, the product extenseffectiveness and intenseffectiveness in group 1 broiler chickens

Table 3
The results of studies of *Eimeria* invasion extensity and intensity and extenseffectiveness and intenseffectiveness of the studied products at different time points during broiler chicken rearing

Group and products	Number of chickens	Extensity and intensity of invasion (%) during testing				Product extenseffectiveness and intenseffectiveness (%) during testing		
		day 20	day 26	day 36	day 46	day 26	day 36	day 46
		EI/II	EI/II	EI/II	EI/II	EE/IE	EE/IE	EE/IE
Test group 1 solicox + chicktonic	50	15.1/ 2.6	14.4/ 2.2	13.7/ 2.1	9.3/ 1.1	74.3/ 93.1	75.9/ 95.6	89.2/ 96.0
Test group 2 maduvet + tylosin	50	16.2/ 2.8	16.0/ 2.5	10.0/ 2.9	6.7/ 2.1	77.2/ 86.9	77.8/ 91.9	87.6/ 94.0
Test group 3 eimeterm 2.5% + enrofloxacin	50	16.8/ 2.6	15.0/ 2.2	11.2/ 2.0	7.3/ 1.5	75.3/ 92.2	76.9/ 95.1	81.4/ 96.0
Control group	50	48.0/ 35.8	56.9/ 48.4	73.2/ 43.8	87.6/ 42.6	–	–	–

were 89.2 and 96%, in group 2 broiler chickens – 87.6 and 94%, in group 3 broiler chickens – 81.4 and 96%, respectively.

By the end of the experiment, the extensity and intensity of invasion in the control group chickens were 87.6 and 42.6%, respectively.

CONCLUSION

The tests performed showed that the clinical signs of eimeriosis were reported in the control group broiler chickens throughout the entire observation period, and in the test group chickens – only until the treatment started. In the control group, 17 chickens died, the survival rate was 66%. Eimeriosis-associated mortality of the control group chickens was 5–6 times higher than that of the test group chickens.

Solicox at a dose of 2 ml per 1 L of drinking water in combination with chicktonic at a dose of 1 ml per 1 L of water for 4–5 days, as well as eimeterm 2.5% given with water at a dose of 7 mg/kg of the body weight (which is equivalent to 1 ml of the product per 1 L of drinking water) in combination with enrofloxacin at a dose of 3 ml per 1 L of drinking water during 5–6 days demonstrated high therapeutic effectiveness against eimeriosis in broiler chickens.

The effectiveness of maduvet given at a dose of 3–5 mg/kg of body weight twice in combination with tylosin at a dose of 5 g of powder per 10 L of drinking water once a day during 5 days was 94%.

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Genetic analysis of nucleotide sequences of neuraminidase gene of highly pathogenic avian influenza A/H5N8 virus isolates recovered in the Russian Federation in 2020

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SUMMARY

Avian influenza is a highly dangerous viral disease that causes huge economic damage to poultry farming. Currently, highly virulent influenza virus with N8 neuraminidase subtype is quite often detected in populations of domestic and wild birds in various countries of the world. The article provides data on complete nucleotide sequences of the neuraminidase gene of highly pathogenic avian influenza virus isolates recovered in the second half of 2020 from pathological material received from four regions of the Russian Federation. The conducted research showed that the subtype of the isolated virus was N8. According to the phylogenetic analysis, isolates of N8 virus belong to group 8C.4. During the phylogenetic analysis of the neuraminidase, we also took into account data on hemagglutinin classification, according to which H5N8 virus isolates belong to a widespread clade 2.3.4.4. Viruses of the clade were first registered in 2010 in China and they have been circulating up to now. The paper also provides data of a comparative analysis of nucleotide sequences of the studied isolates and the isolates from the international GenBank and GISAID databases, recovered in other countries from 2007 to 2020. During the analysis of the amino acid sequence of the studied isolates, no substitutions were found in the positions that affect resistance to neuraminidase inhibitors. The complete nucleotide sequences of the neuraminidase gene of the avian influenza virus subtype N8 (isolates A/domestic goose/OMSK/1521-1/2020, A/duck/Chelyabinsk/1207-1/2020, A/duck/Saratov/1578-2/2020, A/goose/Tatarstan/1730-2/2020) are published in the international GenBank and GISAID databases. Based on the analysis of the nucleotide sequences of the studied isolates, the article shows gradual evolution of the N8 subtype virus.

Keywords: avian influenza virus, RT-PCR, sequencing, N8 neuraminidase subtype, phylogenetic analysis, H5N8

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Генетический анализ нуклеотидных последовательностей гена нейраминидазы изолятов вируса высокопатогенного гриппа птиц A/H5N8, выделенных на территории Российской Федерации в 2020 году

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РЕЗЮМЕ

Грипп птиц является особо опасной болезнью вирусной этиологии, наносящей огромный экономический ущерб птицеводству. В настоящее время в популяциях домашних и диких птиц в различных странах мира достаточно часто выявляют высоковирулентный вирус гриппа с нейраминидазой подтипа N8. В статье представлены данные по изучению полных нуклеотидных последовательностей гена нейраминидазы изолятов вируса высокопатогенного гриппа птиц, выделенных во второй половине 2020 г. из патологического материала, поступившего из четырех регионов Российской Федерации. В результате проведенных исследований определен подтип выделенного вируса – N8. Согласно данным филогенетического анализа, изоляты вируса N8 относятся к группе 8С.4. При проведении филогенетического анализа по нейраминидазе также учитывали данные классификации по гемагглютинину, согласно которой изоляты вируса H5N8 относятся к широко распространенной кладе 2.3.4.4. Вирусы данной клады впервые зарегистрированы в 2010 г. в Китае и продолжают циркулировать до настоящего времени. Также в работе приведены данные сравнительного анализа нуклеотидных последовательностей исследуемых изолятов и изолятов из международных баз данных GenBank и GISAID, выделенных в других странах в период с 2007 по 2020 г. В ходе проведения анализа аминокислотной последовательности исследуемых изолятов в позициях, которые влияют на резистентность к ингибиторам нейраминидаз, замен не обнаружено. Полные нуклеотидные последовательности гена нейраминидазы вируса гриппа птиц подтипа N8 изолятов A/domestic goose/Omsk/1521-1/2020, A/duck/Chelyabinsk/1207-1/2020, A/duck/Saratov/1578-2/2020, A/goose/Tatarstan/1730-2/2020 опубликованы в международных базах GenBank и GISAID. На основании анализа нуклеотидных последовательностей изученных изолятов показана постепенная эволюция вируса подтипа N8.

Ключевые слова: вирус гриппа птиц, ОТ-ПЦР, секвенирование, подтип нейраминидазы N8, филогенетический анализ, H5N8

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INTRODUCTION

For many years, an unfavorable situation with highly pathogenic avian influenza (HPAI) has been observed around the world. Since 1996 highly virulent avian influenza virus subtype H5N1, isolated from domestic geese on one of the farms in Guangdong Province (China), has become epizootic [1]. Due to the disease introduction into the Russian Federation in 2005 resulting in significant damage to the national poultry industry, modern diagnostic methods were developed [2], primarily to detect A/H5N1 virus. From 2016 to 2020, outbreaks of HPAI caused by the H5N8 subtype were regularly recorded in Russia and in many countries of Asia, Africa and Europe.

One of the first HPAI H5N8 cases was reported in 1983, when the virus was detected in turkeys in Ireland [3, 4]. In 2001, the next H5N8 case was detected in wild birds in the State of New Jersey (the USA) during environmental monitoring, and since then several sporadic cases of HPAI have been reported in the country. After three HPAI isolates were detected in Korea in 2014: A/breeder duck/Korea/Gochang1/2014 (H5N8), A/duck/Korea/Buan2/2014 (H5N8) and A/Baikal Teal/Korea/Donglim3/2014 (H5N8), H5N8 subtype has spread widely across the USA, and then throughout other Asian and European countries [5–8]. The pathogenicity index of this virus is significantly higher than that of the virus isolated in Ireland [5]. According to Y. J. Lee et al., highly virulent avian influenza viruses of H5N8 subtype detected in Korea in 2014 occurred as a result of reassortment of the A/duck/Jiangsu/k1203/2010 (H5N8) virus with viruses of other subtypes that circulated in China from 2009 to 2012 [9]. The exact origin of the virus discovered in 2010 in China

remains unknown. K. Zhao et al. note in their studies that this strain was very likely a new reassortant of three subtypes: H5N1 (high identity in the PB1, PA, M, NS genes), H3N8 (in NA gene), H6N2 (NP gene) [10].

In late 2016, HPAI epidemic situation in the Russian Federation deteriorated. Thus, from November to December 2016, HPAI H5N8 outbreaks in domestic birds were recorded in the Astrakhan and Rostov Oblasts, in the Krasnodar Krai and the Republic of Kalmykia. In 2017, more than 30 cases of highly virulent avian influenza virus subtype H5N8 were detected in poultry herds in 8 regions: the Rostov, Moscow, Nizhny Novgorod and Samara Oblasts, the Republics of Tatarstan, Mari El, as well as in the Udmurt and Chechen Republics. Influenza cases were detected in wild migrating birds in the Krasnodar Krai and in the Kaliningrad Oblast, and in zoo birds in the Voronezh Oblast. The disease outbreaks resulted in huge economic losses on commercial farms of the Astrakhan, Rostov, Moscow Oblasts, and in the Republic of Tatarstan [11].

In 2018, HPAI H5N8 was found in domestic birds from the Kursk, Orel, Voronezh, Smolensk, Saratov, Samara, Ulyanovsk, Penza, Nizhny Novgorod, Rostov Oblasts, the Republics of Udmurtia, Mari El, Chuvashia and Tatarstan (more than 80 cases) [12].

Thus, H5N8 influenza viruses, closely related to the isolates originally recovered in South Korea in early 2014, have globally spread. According to the information published by the OIE on November 12, 2020, 265 outbreaks of highly pathogenic avian influenza of H5N8 subtype (111 in domestic, 154 in wild birds) were registered in Europe, Asia and Africa [13], including outbreaks in 12 regions of the Russian Federation: Kostroma, Kurgan, Omsk, Rostov, Samara, Saratov, Tomsk, Tyumen, Chelyabinsk Oblasts, Khanty-Mansi Autonomous Okrug – Yugra, the Republic of Tatarstan, the Karachay-Cherkess Republic.

The purpose of this work was to genetically analyze nucleotide sequences of N8 neuraminidase gene in HPAI virus isolates, recovered in the Russian Federation in 2020, in order to get up-to-date information on the genetic relatedness of HPAI isolates.

MATERIALS AND METHODS

Viruses. The following isolates of the avian influenza virus subtype H5N8 were used in the work:

– A/duck/Chelyabinsk/1207-1/2020 – it was isolated from a domestic duck in the village of Peschanoe, the Uvelsky Raion of the Chelyabinsk Oblast in late July 2020;

– A/domestic goose/Omsk/1521-1/2020 – it was isolated from a domestic goose in the village of Irtyshsky, the Omsk Raion, the Omsk Oblast in September 2020;

– A/duck/Saratov/1578-2/2020 – it was isolated from a domestic duck in the Engels Raion of the Saratov Oblast in September 2020;

– A/goose/Tatarstan/1730-2/2020 – it was isolated from a domestic goose in the village of Meshcheryakovo, the Buinsky Raion of the Republic of Tatarstan in October 2020.

The virus was isolated in 10–11-day-old SPF chicken embryo eggs.

Primers. Several primer systems were used in the work, designed to detect RNA of HPAI subtype N8 and to determine complete nucleotide sequence of the neuraminidase (NA) gene of HPAI subtype N8 isolates (ZAO "Syntol", Russia).

Extraction of RNA. Viral RNA was extracted from the allantoic fluid of SPF chicken embryo eggs infected with the corresponding isolates, using the AmpliPrime RIBO-sorb kit (NextBio LLC, Russia) in accordance with the instructions for use.

Reverse transcription polymerase chain reaction (RT-PCR). Reverse transcription followed by amplification of NA gene fragments to determine the complete nucleotide sequence of HPAI isolates of N8 subtype was performed in BioRad programmable amplifiers (Bio-Rad Laboratories, USA). Synthesis of the first chain of complementary DNA on viral RNA was carried out using RNA-dependent DNA polymerase from avian myeloblastosis virus (AMV Reverse Transcriptase). The reaction was performed in 25 µl of reaction mixture containing 1 µl of 10 mM dNTP, 5 µl of 5× buffer, 3 µl of 25 mM $MgCl_2$, 1 µl of 10 µM forward and reverse primer, 0.125 µl of reverse transcriptase (Promega, USA), and 0.25 µl of Taq polymerase, 8.625 µl of RNase-free water and 5 µl of total RNA. To prevent evaporation of the mixture, 15 µl of Mineral Oil Light White (MP Biomedicals, France) was layered on top. The reaction mixture was incubated in an amplifier at 50 °C for 25 minutes to develop the first cDNA chain. Then they were heated at 95 °C for 10 minutes. The next 39 cycles of conventional PCR were carried out at the following temperature conditions: denaturation at 95 °C – for 50 seconds, annealing of primers at 55 °C – for 50 seconds, elongation at 72 °C – for 1 minute.

The amplification products were analyzed by electrophoresis in 1% agarose gel stained with ethidium bromide.

Purification of PCR products. cDNA fragments amplified in PCR were purified using the Wizard® SV Gel and PCR Clean-Up System kit (Promega, USA) in accordance with the manufacturer's instructions.

DNA sequencing. The primary nucleotide sequence of AIV NA gene fragments was determined in an automatic sequencer ABI Prism 3100 Genetic Analyzer (Applied Biosystems, USA) using the BigDye™ Terminator Cycle Sequencing kit (Applied Biosystems, USA) according to the manufacturer's instructions.

Phylogenetic analysis of nucleotide sequences was performed using the BioEdit Software, Version 7.0.5.3 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). To compare the complete sequences of NA gene of the recovered isolates we used NA gene sequences of the AIV/N8 strains from the international GenBank (http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html) and GISAID databases.

The phylogenetic tree was constructed using the NJ algorithm (including the resampling method "bootstrap") in the implementation of the MEGA package, Version 7.0.26.

RESULTS AND DISCUSSION

One of the main objectives of this research was to obtain up-to-date information about the genetic relatedness of AIV isolates recovered in 2020 in the Russian Federation, as well as their comparison with other AIVs of subtype N8.

At the first stage of the research, we determined nucleotide sequences of the neuraminidase gene of the four influenza virus isolates and compared them with the sequences of AIV N8 subtype from Eurasia and Africa stored in the international databases.

As a rule, the genetic characterization of avian influenza viruses is based on the hemagglutinin classification, which was implemented by the WHO/OIE/FAO working

group and is widely used by the research community. Judging by the available publications, there is no officially regulated classification by neuraminidase. The only publication detailing the classification of neuraminidase subtypes was prepared in 2012 by American scientists from the University of Nebraska [14]. However, since then there have been significant genetic changes in avian influenza viruses, a large mass of new data has been obtained, which raises the question of adding new information to the proposed classification system [15–17]. The introduction of an official virus classification system is not an easy task and it requires a thorough analysis of all the nucleotide sequences of isolate genes available in the database, alignment, construction of phylogenetic trees and, based on them, designation of clades and subclades. An even more difficult question is whether the new system of virus nomenclature will be adopted and used by scientists studying the influenza virus.

According to J. Xu et al., neuraminidase of subtype N8 is divided into three lines, each line has a letter designation: 8A, 8B, 8C [14]. Line 8A is "North American", line 8B is represented by viruses isolated from horses, and the "Eurasian" line belongs to 8C. However, it is clear now that the "line division" approach is not enough to describe the viruses identified over the past few years. For example, this classification does not include viruses of the H5N8 subtype, which were first isolated in China in 2010 and subsequently became widespread in Southeast Asia, and then in Russia, Europe and North America. Therefore, when conducting a phylogenetic analysis of the Russian isolates recovered in 2020 we took into account classification data for both neuraminidase and hemagglutinin described in publications.

For comparative and phylogenetic analyses of nucleotide sequences of NA gene of AIV N8 isolates obtained in this study, we used sequences of known strains of this subtype belonging to different genetic groups from GenBank and GISAID databases. 92 nucleotide sequences of the neuraminidase gene of 1,370 nucleotide bases in size were considered in the work.

A dendrogram was constructed based on the complete sequences of the NA gene of AIV N8 (Fig.), including viruses isolated in the Russian Federation, China, Thailand, Vietnam, Korea, England and other countries from 2007 to 2020. The statistical reliability of the topology of phylogenetic trees was checked using bootstrap analysis; calculations were performed for 500 repetitions.

The Russian isolates of AIV H5N8 presented in the work are highlighted in red font in the figure. The phylogenetic tree constructed on the basis on the nucleotide sequences of NA gene demonstrated four different groups. Based on the classification of neuraminidase developed by J. Xu et al., it can be concluded that all the isolates used in the study belong to the "Eurasian" line 8C, which over time has divided into several separate groups. The identification of groups and subgroups clearly indicated on the tree was performed independently using as an example classification of neuraminidase of other subtypes, also developed by J. Xu et al. [14]. As a result, each group was given a number. According to number Group One was named 8C.1, Group Two – 8C.2, Group Three and Four – 8C.3 and 8C.4 respectively. Groups 8C.1, 8C.2, and 8C.3 included isolates of low pathogenic avian influenza virus.

Group 8C.4 mainly included isolates of highly pathogenic avian influenza virus, i. e. A/domestic goose/Omsk/1521-1/2020, A/duck/Saratov/1578-2/2020, A/duck/Chelyabinsk/1207-1/2020, A/goose/Tatarstan/1730-2/2020.

According to the classification by hemagglutinin, the isolate Group 8C.4 is comparable to Subclade 2.3.4.4.b of Clade 2.3.4.4.

The analysis of avian influenza virus isolates of H5N8 subtype recovered in 2020 demonstrates significantly greater heterogeneity, as compared to the group of isolates detected in 2016–2017. Thus, isolates of A/mute swan/Kazakhstan/1-267-20-B/2020, A/barnacle goose/Germany-SH/AI02167/2020, A/chicken/England/030720/2020, A/chicken/Netherlands/20016597-026030/2020, A/domestic goose/Omsk/1521-1/2020, A/duck/Saratov/1578-2/2020, A/duck/Chelyabinsk/1207-1/2020 form a very homogeneous group of isolates which, presumably, spread due to the migration of wild birds. The difference between these isolates is 0.1–0.6%. However, it is worth noting that isolates A/domestic duck/Poland/285/2020 and A/Mandarin duck/Korea/H242/2020 recovered in Poland and South Korea show significantly greater differences (4.1–4.9%). In addition, detection of isolate A/goose/Tatarstan/1730-2/2020 is of great interest, as it demonstrates a high similarity with avian influenza virus isolates of the same H5N8 subtype isolated in 2017 and differs from most isolates of 2020. The level of differences of A/goose/Tatarstan/1730-2/2020 isolate from other isolates identified in 2020 was 3.3%, and its genetic similarity with A/swan/Voronezh/2/2017 isolate was 0.6%.

A comparative analysis of AIV H5N8 isolates demonstrated that the maximum level of differences in the nucleotide sequence of the NA gene in Group 8C.4 was 9%.

The amino acid sequence of the studied isolates was also analyzed. As indicated in the literature, there are several amino acids, substitutions of which may affect the AIV resistance to drugs inhibiting neuraminidase activity (zanamivir, oseltamivir): E119Q (number 117 based on N8), R292K (291 based on N8), V116D (114 based on N8), or potential sensitivity to oseltamivir and peramivir: H274Y (273 based on N8) [18–20]. There were no substitutions in the predicted amino acid sequence for the above-mentioned positions. Amino acid residues directly involved in the catalytic activity of an enzyme remained unchanged: R118 (116 based on N8), D151 (149 based on N8), R152 (150 based on N8), R292 (291 based on N8).

Comparative and phylogenetic analyses of AIV/N8 isolates indicate the ongoing evolution of the highly pathogenic avian influenza virus of H5N8 subtype in various geographical regions.

The obtained nucleotide sequences of neuraminidase gene of the following AIV N8 isolates A/domestic goose/Omsk/1521-1/2020, A/duck/Chelyabinsk/1207-1/2020, A/duck/Saratov/1578-2/2020, A/goose/Tatarstan/1730-2/2020 were published in international databases GenBank and GISAID (MW276113, EPI1812535, EPI1811679, EPI1815193).

CONCLUSION

The research undertaken helped to determine nucleotide sequences of the NA gene of AIV A/H5N8 isolates: A/domestic goose/Omsk/1521-1/2020, A/duck/Chelyabinsk/1207-1/2020, A/duck/Saratov/1578-2/2020,

Fig. Phylogenetic tree based on the nucleotide sequences of NA gene of HPAI subtype N8

A/goose/Tatarstan/1730-2/2020. Up-to-date information was obtained on genetic relatedness based on NA gene of the studied AIV N8 isolates and isolates recovered from 2007 to 2020 in the Russian Federation and other countries. It was demonstrated that more information shall be added to the current N8 subtype-based classification of avian influenza viruses, due to the wide and rapid spread of genetically similar avian influenza viruses of H5N8 subtype in Europe, Asia, and Africa during 2014–2017.

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Epidemiological monitoring of avian influenza in the Republic of Crimea in 2019–2020

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SUMMARY

The paper presents results of avian influenza epidemiological monitoring in the Republic of Crimea in 2019–2020. The attention was focused on the study of water basins of the Azov and Black Seas, the Sivash Lagoon and freshwater lakes in the Feodosia Urban Okrug, Leninsky, Sovetsky, Nizhnegorsky, Chernomorsky and Saksky Raions to detect the avian influenza virus circulation. Examination of the above mentioned areas showed that some freshwater reservoirs became shallow and dry, and aquatic vegetation degraded. The natural biotope analysis conducted in 2019 and 2020 showed a decreased number of semiaquatic wild birds. The pathological material was sampled from semiaquatic and migratory wild birds, as well as from poultry kept in poultry farms and backyards. The collected samples were tested using real-time RT-PCR. In 2019, the AIV type A (H9) genome was detected in one fecal sample taken from wild birds near Kuchuk-Adzhigol Lake in Feodosia Urban Okrug. The AIV type A (H5) genome was detected in 2020 during laboratory testing of pathological material taken from the remains of a mute swan within the shoreline of a freshwater lake near the Ermakovo settlement of the Dzhankoysky Raion. The genetic analysis was performed in the FGBI "ARRIAH" (Vladimir), and the N8 subtype neuraminidase of the influenza virus isolate was determined. The comparative genetic analysis of 258 bp nucleic acid sequences of the AIV H gene fragment showed that the identified isolate belongs to the Asian genetic lineage of highly pathogenic AIV subtype H5 (clade 2.3.4.4) associated with the epidemic spread in Asia, Europe, the Middle East and Africa in 2016–2020.

Keywords: wild migratory birds, semiaquatic birds, avian influenza, migration, bird fauna, epidemiological monitoring

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Эпизоотологический мониторинг гриппа птиц на территории Республики Крым в 2019–2020 гг.

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РЕЗЮМЕ

В статье представлены результаты эпизоотологического мониторинга гриппа птиц на территории Республики Крым в 2019–2020 гг. При изучении циркуляции вируса гриппа птиц особое внимание уделено акваториям Азовского и Черного морей, заливу Сиваш и пресноводным озерам городского округа Феодосия, Ленинского, Советского, Нижнегорского, Черноморского и Сакского районов. При обследовании вышеуказанных районов было отмечено обмеление и пересыхание некоторых пресноводных водоемов, деградация водной растительности. Анализ состояния обследуемых природных биотопов за 2019 и 2020 гг. показал снижение численности диких околоводных птиц. Произведен отбор проб патологического материала от околоводной и дикой перелетной птицы, а также от птиц, содержащихся на птицефабриках и в личных подсобных хозяйствах граждан. Полученные образцы исследовали методом полимеразной цепной реакции в реальном времени с обратной транскрипцией. В 2019 г. в одной пробе помета от дикой птицы с озера Кучук-Аджиголь городского округа Феодосия обнаружен геном вируса гриппа А птиц подтипа H9. В результате проведенных в 2020 г. лабораторных исследований патологического материала (останки лебедя-шипуна), отобранного на береговой линии пресноводного озера вблизи с. Ермаково Джанкойского района, выявлен геном вируса гриппа А подтипа H5. На базе ФГБУ «ВНИИЗЖ» (г. Владимир) был проведен генетический анализ и определен подтип нейраминидазы выделенного изолята вируса гриппа – N8. Сравнительный генетический анализ нуклеиновых последовательностей фрагмента гена H длиной 258 н. о. вируса гриппа птиц показал, что выявленный изолят принадлежит к азиатской генетической линии вируса высокопатогенного гриппа птиц подтипа H5 (клада 2.3.4.4), получившего эпизоотическое распространение в 2016–2020 гг. в странах Азии, Европы, Ближнего Востока и Африки.

Ключевые слова: дикая перелетная птица, околоводная птица, грипп птиц, миграция, орнитофауна, эпизоотологический мониторинг

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INTRODUCTION

Avian influenza (AI) is a highly contagious viral disease of birds, subject to the World Organisation for Animal Health (OIE) notification. It causes enormous economic damage and poses a serious threat to poultry farms around the globe. The causative agent is an RNA virus belonging to the family *Orthomyxoviridae*, type A (*Influenza A*). There are 16 known hemagglutinin antigens (H1–H16) and 9 neuraminidase antigens (N1–N9). The AI agent has unique abilities to change its antigenic structure and cross the species barrier [1–8].

Avian influenza virus (AIV) is steadily circulating in wild migratory bird populations in many countries. Aquatic and semiaquatic birds are the primary natural reservoir of the virus [1, 2, 7–9]. The Republic of Crimea located at the crossroads of wild bird migration routes from Asia to European and African countries and back, is a stopover and wintering place for many wild semiaquatic bird species. It was primarily the AIV subtype H5N1 known to be circulating until 2014 and causing epidemics in the Crimea in 2005, 2006 and 2008 [5, 10, 11]. At present the AIV subtype H5N8 has become globally widespread and was detected in domestic and wild bird populations in Europe, Asia and Africa. In the Russian Federation the highly pathogenic H5N8 influenza virus was initially isolated from dead wild birds at Uvs Nuur Lake (Republic of Tyva) in May 2016 [2, 6–8, 9, 11–15]. Then the disease became widespread and was recorded in both wild and domestic birds in the immediate vicinity of the Crimean Peninsula, namely in the Rostov Oblast, Krasnodar Krai, as well as in the Kherson and Odessa Oblasts in Ukraine [3, 16]. The next epizootic wave started in August 2020 in the Southern Urals and Western Siberia and affected most of the European countries by the end of the year. The current epidemic is caused by H5N8, H5N5, H5N4, H5N3, H5N1 subtype viruses belonging to genetic clade 2.3.4.4, which originates from the Middle East [6–8].

From 2009 to the present time no AI disease and mortality cases have been reported in backyard and commercial poultry on the territory of Crimea. But taking into account that the AIV (H5N8) genome was detected in a mute swan in the territory of the peninsula in 2017, and the animal products imported from the Penza Oblast [11, 16] tested positive for AI (subtype H9) in 2018, there remains a real threat of AIV introduction into backyard and commercial flocks in the Republic of Crimea.

Thus, the AI epidemiological monitoring is of relevant importance on the territory of the Republic of Crimea.

MATERIALS AND METHODS

The tests were conducted on the basis of the Laboratory for Molecular Diagnostics of the Laboratory and Diagnostic Center of the FGBI "ARRIAH" Branch in the Republic of Crimea in 2019–2020.

Biological material was collected from wild birds during expeditions to the coast of the Azov and Black Seas, the Sivash Lagoon and freshwater lakes in the Feodosia Urban Okrug, Leninsky, Sovetsky, Nizhnegorsky, Chernomorsky and Saki Raions with participation of the Rosselkhoznadzor Administration for the Republic of Crimea and Sevastopol City (Rosselkhoznadzor) specialists. The expeditions were arranged pursuant to the research work plan of the FGBI "ARRIAH" Branch in the Republic of Crimea and state epidemiological monitoring plans.

The species identification of wild birds subjected to biomaterial sampling was carried out using a bird field guide [17]. During the expeditions the state of natural biotopes was assessed and the number of bird fauna in this territory was calculated.

The material was delivered to the laboratory in sealed water-proof insulated containers with cool packs in compliance with current regulations. Laboratory tests were carried out using Russia-manufactured commercial test kits in accordance with the Veterinary Rules for Avian Influenza A Laboratory Diagnosis [18].

Biomaterial samples from wild birds shot in various hunting areas of the Crimea, samples from poultry kept in poultry farms and backyards, as well as samples of animal products were sent to the laboratory of the FGBI "ARRIAH" Branch in the Republic of Crimea.

Identification of the AIV genome was carried out by extraction of total nucleic acid from pathological material, reverse transcription of the obtained RNA and amplification of specific sites of the cDNA derived from the influenza A virus using real-time polymerase chain reaction (real time RT-PCR). The following was used: a GRIPP commercial test

Table 1
Pathological material sampling from wild birds in 2019

Sampling date	Type of pathological material	Number of samples	Sampling site
February 18, 2019	Faeces (wild birds)	1	6.5 km from Znamenskoye settlement (Spit Belyaus), Lake Donuzlav shore (45.346111, 32.954625)
	Eurasian coot (*Fulica atra*), Gull (*Larus*) (carcasses)	4	
	Faeces (wild birds)	1	Sasyk-Sivash Lake, Yevpatoria (45.200887, 33.415658)
	Wild birds (carcasses)	9	Panskoe Lake, Chernomorsky Raion
August 01, 2019	Faeces (wild birds)	5	3 km from Yermakovo settlement, Dzhankoysky Raion
	Gull (*Larus*) (remains)	1	
	Faeces (wild birds)	25	5 km from Yermakovo settlement, Dzhankoysky Raion, shore of Sivash Lagoon
	Faeces (wild birds)	3	5 km from Tomashevka settlement, Dzhankoysky Raion, Aigulskoye Lake
	Faeces (wild birds)	8	3 km east of Tselinnoye settlement, Dzhankoysky Raion
	Faeces (wild birds)	10	3 km from Melkovodnoye settlement, Dzhankoysky Raion, Aigulskoye Lake
August 08, 2019	Faeces (wild birds)	1	area of the Ablyamitsky bridge, Lake Donuzlav
	Faeces (wild birds)	12	5 km from Znamenskoye settlement, Lake Donuzlav
	Faeces (wild birds)	6	5 km from Mezhvodnoye settlement, Lake Panskoye, Chernomorsky Raion
	Faeces (wild birds)	7	5 km from Yevpatoria, Sasyk-Sivash Lake
	Sparrow (*Passeridae*) (carcass)	1	
	Gull (*Larus*) (remains)	1	
October 18, 2019	Mute swan (*Cygnus olor*) (remains)	1	Sasyk-Sivash Lake (45.204513, 33.410529)
	Mute swan (*Cygnus olor*) (remains)	1	Sasyk-Sivash Lake (45.204128, 33.411204)
	Faeces (wild birds)	4	Sasyk-Sivash Lake
	Faeces (wild birds)	18	shore of Lake Donuzlav, Ablyamitsky bridge
	Faeces (wild birds)	15	shore of Lake Donuzlav, Spit Belyaus, 5 km from Znamenskoye settlement
November 25, 2019	Faeces (wild birds)	27	Lake Kuchuk-Ajigol, Feodosia Urban Okrug
	Total	161	

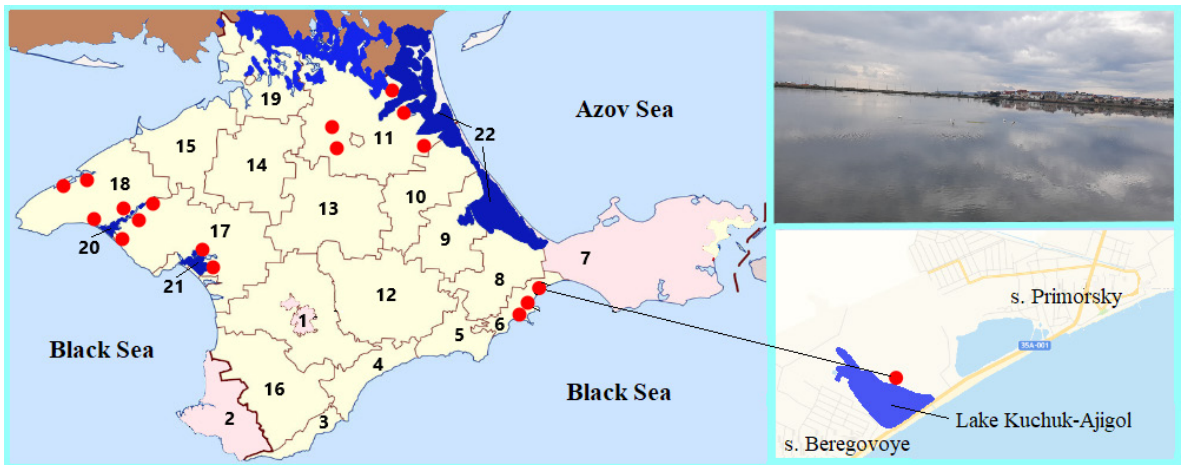

Fig. 1. Sampling of pathological and biological material from semiaquatic wild birds in 2019

● – location of sites for pathological and biological material sampling from wild birds

1. Simferopol
2. Sevastopol
3. Yalta
4. Alushta
5. Sudak
6. Feodosia
7. Leninsky Raion
8. Kirovsky Raion
9. Sovetsky Raion
10. Nizhnegorsky Raion
11. Dzhankoysky Raion
12. Belgorodsky Raion
13. Krasnogvardeysky Raion
14. Pervomaysky Raion
15. Razdolnensky Raion
16. Bakhchisaraysky Raion
17. Saksky Raion
18. Chernomorsky Raion
19. Krasnoperekopsky Raion
20. Donuzlav Lake
21. Sasyk-Sivash Lake
22. Sivash Lagoon

kit (the Rospotrebnadzor Central Research Institute of Epidemiology, Russia) for PCR detection and differentiation of influenza virus (*Influenza A virus*) and differentiation of subtypes (A H5, H7, H9), which includes AmpliPrime RIBO-Sorb reagents for RNA extraction, Reverta-L for the reverse transcription and reagents for *Influenza A virus* cDNA amplification and subtypes A H5, H7, H9 differentiation using real-time hybridization and fluorescence detection. The fluorescent signal was detected using the Rotor-Gene Q amplifier (Qiagen, Germany).

RESULTS AND DISCUSSION

In 2019, the FGBI "ARRIAH" branch of the Republic of Crimea together with the Rosselkhoznadzor professionals arranged 5 expeditions, during which 8 Crimean raions were surveyed (Fig. 1) and 161 samples of pathological material from wild birds were collected (Table 1). The geographical sampling distribution is shown in Figure 1.

Wild waterfowl mass deaths were registered at Lake Donuzlav in February 2019. At the time significant congregations of Eurasian coots (*Fulica atra*) (about 8–10 thousand) were observed in this reservoir area. There were also gulls (*Larus*), ducks (*Anatinae*) and single mute swans (*Cygnus olor*) identified. A large number of Eurasian coot (*Fulica atra*) remains (about 1.5–2.0 thousand) (Fig. 2*a*, *b*) and, occasionally, remains of mallards (*Anas platyrhynchos*), common shelducks (*Tadorna tadorna*), black-necked grebes (*Podiceps nigricollis*), Caspian gulls (*Larus cachinnans*) (Fig. 2*c*) were found within the shoreline area.

In addition, Eurasian coots with clinical signs characteristic of the AI acute form and demonstrating lack of movement coordination, neck and head twisting (Fig. 3) were found on the shore [19–21].

No pathological and anatomical findings were observed during necropsy of Eurasian coot carcasses.

Nine wild bird carcasses were found in the Chernomorsky Raion at Lake Panskoye located 20 km from Lake Donuzlav (Table 1): Eurasian coot (*Fulica atra*) – 2, red-crested pochard (*Netta*) – 1, mallard duck (*Anas platyrhynchos*) – 4, Eurasian wigeon (*Mareca penelope*) – 1, greater scaup (*Aythya marila*) – 1. No wild bird mortality was recorded in other Crimean Raions. A decrease in the number of wild migratory birds was also noted during the field expeditions as compared to the previous years, especially on the Sivash Lagoon shore.

Fig. 2. Remains of Eurasian coots (Fulica atra) (a, b) and a Caspian gull carcass (c) at the Donuzlav Lake

Fig. 3. Eurasian coots demonstrating lack of movement coordination, head and neck twisting

A total of 13 samples of pathological material and 2 samples of wild bird faeces were taken during the expedition in February 2019 (Table 1). The AIV genome was not detected in these samples.

In August 2019 two field expeditions were arranged to the sites of semiaquatic bird congregation, during which the reservoirs of the Dzhankoysky, Chernomorsky and Saksky Raions of the Crimea were surveyed (Table 1). Testing of 77 faecal samples from wild birds and 3 pathological material samples (bird remains) showed absence of the AIV genome. During the survey of the above-mentioned areas it was noted that freshwater reservoirs became shallow and some of them dried up completely, degradation of aquatic vegetation was observed everywhere.

The freshwater lakes of the Chernomorsky and Saksky Raions, including the coast of the Karkinitsky Gulf of the Black Sea, were surveyed during the expedition conducted in October 2019. Two pathological material samples from a mute swan and 37 faecal samples from wild semiaquatic birds were collected (Table 1). The AIV genome was not detected in these samples.

The freshwater lakes in the Feodosiya Urban Okrug, Leninsky and Sovetsky Raions, as well as in the Sivash Lagoon area were surveyed on November 25, 2019. Wild semiaquatic birds were observed only at Lake Kuchuk-Ajigol in Feodosia Urban Okrug (Fig. 1). The real-time RT-PCR testing of 27 faecal samples collected in this territory showed that the influenza A subtype H9 virus genome was detected only in one of them.

In 2019 the Rosselkhoznadzor professionals also submitted 291 samples from birds shot in hunting areas and 548 samples from poultry from different Crimean Raions to the Laboratory and Diagnostic Center of the FGBI "ARRIAH" branch (the Republic of Crimea). The AIV genome was not detected in these samples.

In total 1,000 samples of pathological material were tested in the Laboratory for Molecular Diagnostics in 2019, among them 16.1% were samples obtained from wild semiaquatic birds, 29.1% – from wild birds shot in hunting areas and 54.8% – from poultry.

In 2020, the FGBI "ARRIAH" Branch in the Republic of Crimea, together with the Rosselkhoznadzor professionals organized 3 field expeditions (in winter-spring and autumn-winter periods) to wild waterfowl congregation sites, during which the following areas of the Crimean peninsula were surveyed: the shore of the Sivash Lagoon, freshwater lakes of the Feodosiya Urban Okrug, as well as Leninsky, Sovetsky, Nizhnegorsky, Dzhankoy raions (Fig. 4).

Fifty four samples from wild birds were collected for real-time RT-PCR testing, including 2 pathological material samples (the remains of a mute swan – Fig. 5*a* and a saker falcon – Fig. 5*b*) and 52 faecal samples from wild birds.

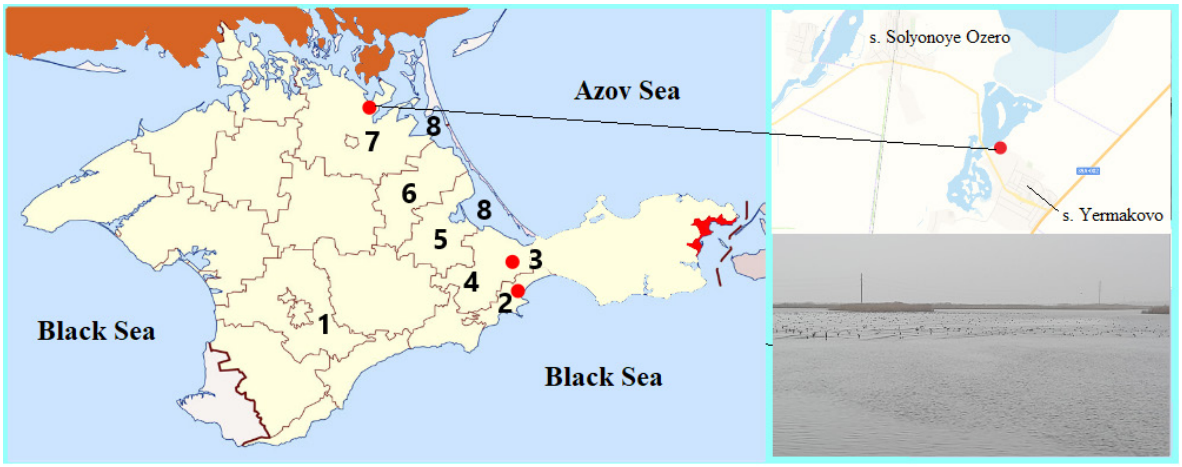

Fig. 4. Pathological and biological material sampling from semiaquatic wild birds in 2020

● *– location of sites for pathological material sampling from wild birds*

1. Simferopol
2. Feodosia Urban Okrug
3. Leninsky Raion
4. Kirovsky Raion
5. Sovetsky Raion
6. Nizhnegorsky Raion
7. Dzhankoysky Raion
8. Sivash Lagoon

The samples were taken on the shore of Lake Kuchuk-Ajigol (Feodosia Urban Okrug), at the Frontovoye reservoir (Leninsky Raion) and the freshwater lake shore near the Yermakovo settlement (Dzhankoysky Raion) (Table 2). No bird congregations were found in the other surveyed areas. Compared to 2019, the number of wild semiaquatic birds decreased, most likely, due to the decomposition of reedbeds, a decrease in the food supply for wild birds, changes in wintering and stopover sites during migration and nesting. The same trend was observed in previous years [4].

Table 2 presents data on the sampling site of pathological and biological material from wild semiaquatic birds, the number of samples taken, as well as the types and numbers of birds observed during field expeditions.

The genome of influenza A virus subtype H5 was detected in the pathological material collected from the remains of a mute swan in the shoreline area of a freshwater lake near the Yermakovo settlement, Dzhankoysky Raion, in December 2020 (Fig. 5*a*).

Fig. 5. Remains of a mute swan (a) and a saker falcon (b)

Table 2
Number and species of semiaquatic wild birds, number of samples in 2020

Sampling date	Bird species observed in the area	Number of birds	Number of collected samples
Frontline reservoir, Leninsky Raion			
February 04, 2020	Grey goose (*Anser anser*)	24	27
	White-fronted goose (*Anser albifrons*)	20	
	Black-necked grebes (*Podiceps nigricollis*)	200	
	Large cormorant (*Phalacrocorax carbo*)	70	
	Saker Falcon (*Falco cherrug*)	1	
Lake Kuchuk-Ajigol, Feodosia Urban Okrug			
November 03, 2020	Mute swan (*Cygnus olor*)	60	21
	Eurasian coot (*Fulica atra*)	400	
	Great bittern (Botaurus *stellaris*)	3	
	Grey heron (*Ardea cinerea*), Great egret (*Ardea alba*)	25	
	Red-headed pochard duck (*Aythya ferina*)	200	
	Greater scaup (*Aythya marila*)	15	
	Tufted duck (*Aythya fuligula*)	20	
	Northern shoveler (*Spatula clypeata*)	90	
	Eurasian teal (*Anas crecca*)	120	
	Gadwall (*Mareca strepera*)	38	
	Mallard duck (*Anas platyrhynchos*)	70	
The shore of a freshwater lake near the Yermakovo settlement, Dzhankoysky Raion			
December 18, 2020	Mute swan (*Cygnus olor*)	18	6
	Mallard duck (*Anas platyrhynchos*)	25	
	Eurasian coot (*Fulica atra*)	300	
		Total	54

Table 3
Number and location of biological material samples taken from poultry, pathological material samples taken from wild birds and animal product samples in 2020

Name of the material	Number of samples	Sampling site
Biological material (chicken droppings)	50	Yalta Urban Okrug, Belogorsky Raion
Biological material (chicken droppings)	50	Pervomaisky Raion
Biological material (chicken droppings)	200	Nizhnegorsky Raion
Biological material (chicken droppings)	25	Simferopol Raion
Biological material (chicken droppings)	100	Razdolnensky Raion
Biological material (chicken droppings)	150	Sudak city, Feodosia city, Feodosia Urban Okrug
Biological material (chicken droppings)	100	Kirovsky Raion
Biological material (chicken droppings)	100	Alushta Urban Okrug
Poultry biological material (cloacal swabs)	50	Yevpatoria Urban Okrug
Biological material (chicken droppings)	75	Sevastopol districts
Pathological material (wild bird carcasses)	14	Hunting areas in Sevastopol city
Pathological material (wild bird carcasses)	9	Hunting areas in Sevastopol city and Leninsky Raion
Pathological material (great bustard *Otis tarda* carcasses)	5	Hunting areas in Leninsky Raion
Frozen poultry meat (turkey) and frozen poultry offal (turkey)	12	Simferopol
Chicken eggs (30 pcs.)	6 pooled samples	Sevastopol city
Biological material (semiaquatic waterfowl faeces)	36	Lake Kuchuk-Ajigol, Feodosia Urban Okrug
Wild bird remains (Saker falcon)	1	
Biological material (semiaquatic waterfowl faeces)	11	Frontline reservoir, Leninsky Raion
Wild bird remains (a mute swan)	1	The shoreline of a freshwater lake near Yermakovo settlement, Dzhankoysky Raion
Biological material (semiaquatic waterfowl faeces)	5	
Total	1,000	

The genetic analysis was performed and the neuraminidase subtype (N8) of the identified influenza virus isolate was identified on the basis of the FGBI "ARRIAH" (Vladimir). The comparative genetic analysis of the AIV nucleic sequences of the 258 bp H gene fragment showed that the identified isolate belongs to the Asian genetic lineage of the highly pathogenic AIV subtype H5 (clade 2.3.4.4), which epidemically distributed in the countries of Asia, Europe, the Middle East and Africa in 2016–2020. According to the GenBank international sequence database, the A/duck/Egypt/SMG4/2019 (H5N8) virus isolated in Egypt in 2019 is the most genetically similar to the identified isolate (99.2–99.6% similarity). In addition, this isolate is genetically similar to H5N8 subtype AIVs detected in Omsk, Kurgan and Chelyabinsk Oblasts in 2020 (98.8–100.0% similarity), and H5N8 subtype AIVs isolated in 2016–2019 in the Voronezh, Rostov, Moscow Oblasts and the Republic of Kalmykia (97% similarity). The hemagglutinin cleavage site in the detected virus is -REKRRKR- which is characteristic of highly virulent AI pathogens. No AIV genome was detected in all the other tested samples.

Additionally, 28 samples collected from birds shot in hunting areas of the Leninsky Raion and Sevastopol city raions, 900 samples of biomaterial taken from poultry in the Belogorsky, Pervomaisky, Nizhnegorsky, Simferopolsky, Razdolnensky, Kirovsky Raions, the cities of Sudak, Yalta, Feodosia and the Urban Okrugs of Yalta, Feodosia, Sevastopol, as well as 18 samples of animal products marketed in Simferopol and Sevastopol were submitted to the Laboratory and Diagnostic Centre of the FGBI "ARRIAH" branch in the Republic of Crimea by the Rosselkhoznadzor professionals (Table 3). The AIV genome was not detected in these samples.

In total, 1,000 samples of pathological and biological material were tested for avian influenza using real time RT-PCR in 2020, out of them 8.2% were samples obtained from wild birds, 90% – from backyard poultry and 1.8% were samples of animal products.

CONCLUSION

Within the AI epidemiological monitoring in the territory of the Republic of Crimea in 2019–2020 nine areas were surveyed and 2,000 samples of pathological and biological material were tested, including 215 samples (10.75%) from wild semiaquatic birds, 319 samples (15.95%) from birds hunted in the hunting areas,

1,448 samples (72.4%) from poultry, 18 samples (0.9%) of products of animal origin.

The AIV genome was detected in two samples (0.1%) collected from wild birds: on the shore of Lake Kuchuk-Ajigol in Feodosia Urban Okrug (avian influenza A subtype H9) in November 2019 and on the lakeshore near the Yermakovo settlement, Dzhankoy Raion (avian influenza A subtype H5) in 2020. This fact is another confirmation that wild migratory birds present the main threat of AI introduction into the Crimean territory.

Therefore, a timely analysis of monitoring study results will allow monitoring the AI epidemic situation and, in case of a threat, taking timely measures to prevent the AIV spread, contain outbreaks and carry out measures aimed primarily at breaking the transmission chain.

The prospect of further research is to conduct consistent and detailed AI epidemiological monitoring on the Crimean peninsula with the coverage expansion of the studied areas (hunting areas, preserve areas).

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Comparative effectiveness of liquid and freeze-dried vaccines for oral vaccination of dogs against rabies

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SUMMARY

Currently, vaccination is the main measure to combat rabies in domestic and wild animals. Inactivated and live vaccines are used for this purpose. Oral vaccines for wild carnivores contain live attenuated rabies viruses in liquid or freeze-dried form, which are packaged inside edible baits. Since there are no consistent data showing advantages of liquid and freeze-dried oral products for vaccine-induced immunity against rabies in animals, we compared effectiveness of these rabies vaccines produced from rabies virus strain VRC-RZ2. Immunogenicity was tested in mongrel dogs aged 3 months and older that are seronegative for rabies virus antigens. The animals were randomly divided into three groups: two experimental and one control group. Group One was fed a block-type bait containing a blister with liquid virus-containing suspension, Group Two was given a block-type bait containing a gelatin capsule with freeze-dried virus suspension. On Day 21 post vaccination, blood samples were taken from all the animals and the obtained sera were examined in virus neutralization test to measure virus neutralizing antibodies titers. The level of the immune response against rabies in the vaccinated dogs was assessed by intracerebral infection of animals with virulent rabies virus strain CVS. The carried out research demonstrated that both groups of the vaccinated dogs had approximately the same titers of virus neutralizing antibodies that ranged from 3.25 to 4.33 $\log_2$. The virus neutralizing antibodies observed in the immunized dogs ensured good protection from virulent CVS strain. All animals of the control group died after infection demonstrating clinical signs of paralytic rabies. The results obtained show that both forms of the oral rabies vaccines are effective.

Keywords: rabies virus, oral vaccine, liquid vaccine, freeze-dried vaccine, antibodies, immunity

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Сравнительная эффективность вакцин в жидкой и лиофилизированной формах при оральной вакцинации собак против бешенства

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РЕЗЮМЕ

В настоящее время основной мерой борьбы с бешенством является вакцинопрофилактика домашних и диких животных, для этого используют инактивированные и живые препараты. Вакцины для оральной иммунизации диких плотоядных животных содержат живые аттенуированные вирусы бешенства, которые в жидком или лиофилизированном виде помещают внутрь съедобной приманки. В связи с отсутствием однозначных данных о преимуществах жидкого и лиофилизированного перорального препарата при формировании у животных поствакцинального иммунитета против бешенства было проведено сравнительное изучение эффективности данных антирабических вакцин, изготовленных из штамма VRC-RZ2 вируса бешенства. Иммуногенную эффективность изучали на беспородных, серонегативных к антигенам вируса бешенства собаках в возрасте от 3 мес. и старше. Животных случайным образом разделили на три группы: две опытные и контрольную. В первой группе собакам скармливали брикет-приманку, содержащую блистер с жидкой вируссодержащей суспензией, во второй группе – брикет-приманку, внутрь которой помещена желатиновая капсула с лиофилизированной суспензией вируса. Через 21 сут после иммунизации у всех животных отбирали пробы крови, полученные из них сыворотки исследовали в реакции нейтрализации для определения титров вируснейтрализующих антител. Напряженность антирабического иммунитета у вакцинированных собак оценивали путем интрацеребрального заражения животных вирулентным вирусом бешенства штамма CVS. В результате проведенного исследования установлено, что в обеих группах иммунизированных собак титры вируснейтрализующих антител были примерно одинаковыми и находились в диапазоне от 3,25 до 4,33 $\log_2$. Выработанные у иммунизированных собак вируснейтрализующие антитела обеспечивали надежную защиту от вирулентного вируса штамма CVS. Все животные контрольной группы после заражения погибли с клиническими признаками паралитической формы бешенства. Полученные результаты свидетельствуют об эффективности обеих форм оральных вакцин против бешенства.

Ключевые слова: вирус бешенства, пероральная вакцина, жидкая вакцина, лиофилизированная вакцина, антитела, иммунитет

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INTRODUCTION

Rabies is a viral disease affecting central nervous system of mammals, including humans [1]. Currently, this disease is endemic in more than 150 countries around the world. Despite the fact that rabies is preventable, according to the World Health Organization (WHO), 59 thousand people die from it every year in the world, mainly in the poorest and most vulnerable communities. About 40% of the victims are children under the age of 15 living in Asia and Africa [2].

The global response to rabies has been fragmented and poorly coordinated so far. Currently, a collective initiative "Unite Against Rabies" is being implemented under the WHO, the goal of the initiative is to achieve zero human deaths from rabies transmitted by dogs by 2030 [3]. A set of joint actions of the the CIS Member States was adopted at the meeting of the CIS Council of Heads of State in 2018 to prevent and control rabies for the period up to 2025 [4].

Wildlife rabies control tactics have changed critically over the past few decades, partially due to the latest scientific discoveries. Historically, rabies control measures mainly consisted in destruction of target animal species [5]. However, scientific breakthroughs have made it possible to develop a method of oral immunization of wild animals by packaging rabies vaccine inside edible baits for carnivores.

There are now various types and forms of baits. Most of them have approximately the same structure and consist of a blister with a vaccine packaged inside a tasty bait, with slight variations in size, bait compositions and types of blisters [6, 7].

Most viruses used in production of live oral rabies vaccines originate from the attenuated Evelyn-Rokitnicki-

Abelseth (ERA) strain, which was derived from the original Street-Alabama-Dufferin (SAD) strain. The parental SAD strain was isolated from the salivary glands of a rabies-infected dog in the USA, in 1935, and then attenuated by serial passages in mice, chicken embryos and various cell lines and was renamed as ERA [8]. The modified SAD Bern vaccine strain was obtained as a result of serial passages of the ERA strain in cell cultures, it was used in the first trials of an oral rabies vaccine in Switzerland [9].

The success of wildlife rabies control by oral immunization has been demonstrated in a number of European countries, such as Estonia, France, Italy and Switzerland. It should be noted that these countries were declared rabies-free only after several years of oral vaccination campaigns using baits containing SAG2 vaccine strain (France also used the recombinant V-RG vaccine) [10–12].

SAG2 (SAD Avirulent Gif) rabies virus strain is a modified live virus selected from SAD Bern in 1990 as a result of two successive mutations [13].

Canada and the USA demonstrated successful use of recombinant vaccines based on vaccinia virus and human adenovirus serotype 5: RABORAL V-RG® (Boehringer Ingelheim Animal Health Inc., USA) and ONRAB® (Artemis Technologies Inc., Canada) for immunization of wild animals [14–17]. These vaccines were produced on the basis of a viral vector, which was created in 1984 as a recombinant vaccinia virus V-RG expressing G-protein gene of ERA rabies virus strain [11, 12, 18].

There are also freeze-dried oral rabies vaccines. One of them was developed by scientists from VNIIVVIM (Russia)[1], the product contains a fixed rabies virus strain TS-80, obtained in 1980 by G. A. Safonov et al. and deposited in the VGNKI on February 17, 1988 [19]. RABIGEN® SAG2 vaccine, developed by Virbac laboratory scientists (France), is another means of specific prevention that has been used in practice. This product is a live modified attenuated rabies vaccine based on recombinant SAG2 rabies virus strain selected from SAD Bern strain during a two-stage amino acid mutation using neutralizing monoclonal antibodies. The effectiveness of RABIGEN® SAG2 has been demonstrated in accordance with the EU requirements for red fox and raccoon dog in Estonia, France, Italy and Switzerland [10].

Despite the fact that the effectiveness of oral immunization of wild carnivores was experimentally confirmed, in order to develop and improve vaccines it is critically important to understand how vaccine viruses penetrate into the host cell and replicate there.

In order to prevent rabies in the field, many countries use blisters with a vaccine (filled with virus-containing suspension prepared from different virus strains). However, some researchers have noted that the distribution of block-type baits in cold weather can make the main immunogenic component freeze, therefore, when the bait is eaten by animals, the vaccine does not have contact with mucous membrane of the oral cavity, but gets into the stomach, thus reducing effectiveness of vaccination [20]. A possible solution to this problem is to use the vaccine with an acid-protective coating that protects the virus from the inactivating effects of gastric juice, or to use freeze-dried oral vaccine that is stable at low temperatures and does not require chewing for the vaccine virus to have contact with the oropharyngeal mucosa [21, 22].

The aim of the study is to compare immunogenic effectiveness of liquid and freeze-dried rabies vaccine produced from rabies virus strain VRC-RZ2.

MATERIALS AND METHODS

The vaccine strain of the virus. Fixed rabies virus strain VRC-RZ2 was obtained from an organ/tissue rabic isolate (puppy brain) and deposited in the collection of microorganisms of the RSE "Research Institute for Biological Safety Problems" (RSE "RIBSP", Kazakhstan) with the registration number P-7-04/D. This strain is recommended for production of rabies vaccine used for oral immunization of animals (Patent RK No. 17453[2]). The titer of the vaccine strain is 6.0–6.5 lg MLD_{50}/0.03 cm^3.

Vaccine. Oral vaccines from VRC-RZ2 strain were used for the research. The vaccines were used in two forms:

– liquid – the product weighing – 25–30 g contains 10 cm^3 of rabies virus suspension in a blister packaged in a block-type bait. The virus titer in one vaccine dose is $10^{6.75}$ $TCID_{50}$/cm^3.

– freeze-dried – the product weighing – 25–30 g contains 10 cm^3 of freeze-dried suspension of rabies virus with a stabilizing and acid-resistant polymer in a gelatin capsule packaged in a block-type bait. The virus titer in one vaccine dose is 10^7 $TCID_{50}$/cm^3.

Challenge virus. Reference rabies virus strain CVS was used in the experiment. It is maintained and stored in the collection of microorganisms of the RSE "RIBSP" (Kazakhstan). The infectious activity of the virus is 4.5–5.0 MLD_{50}.

Animals and preparing them for the experiment. 15 mongrel dogs aged 3 months and older were used in the research.

Before the experiment started, the animals were identified and quarantined for 14 days. During the quarantine they were dewormed, subjected to clinical examination and their sera were tested for specific antibodies to rabies virus in virus neutralization test (VNT) [23]. For the purposes of the experiment, we used those dogs who had no specific antibodies to rabies virus and had not been previously vaccinated against this disease.

Experiments on animals were carried out in accordance with national and international laws and regulations on protection and welfare of animals. The protocol was approved by the Ethical Committee on Animal Experimentation of RSE "RIBSP" of the Committee of Science of the Ministry of Education and Science of the Republic of Kazakhstan (Authorization Number: 0701/20).

Experiments on animals. At the end of the quarantine, the dogs were kept without food for one day, then they were randomly divided into three groups (5 dogs in each). Animals of Group One were fed a block-type bait with liquid vaccine in a blister, dogs of Group Two were fed a block-type bait with freeze-dried vaccine in a gelatin capsule. Group Three was used as a control. On Day 21 post

[1] Khripunov E. M., Isakova N. B., Evseeva S. D., Vishnyakov I. F., Nedosekov V. V., Zhesterev V. I., et al. Virus vaccine against rabies for oral immunization of carnivores. Patent No. 2157700 Russian Federation, MPK A61K 39/205(2006.01), C12N 7/00(2006.01). VNIIVViM. Application 25.01.1999. Publ. 20.10.2000.

[2] Rusanova A. M., Zhilin Ye. S., Troitsky Ye. N., Mamadaliev S. M., Barakbayev K. B., Demchenko A. G. Fixed rabies virus strain VRC-RZ2 for preparation of preventive and diagnostic products. Patent No. 17453 Republic of Kazakhstan, MPK C12N 7/00, C12R 1/93, A61K 39/205. Application 10.12.2004. Publ. 15.12.2009. Bulletin No. 12.

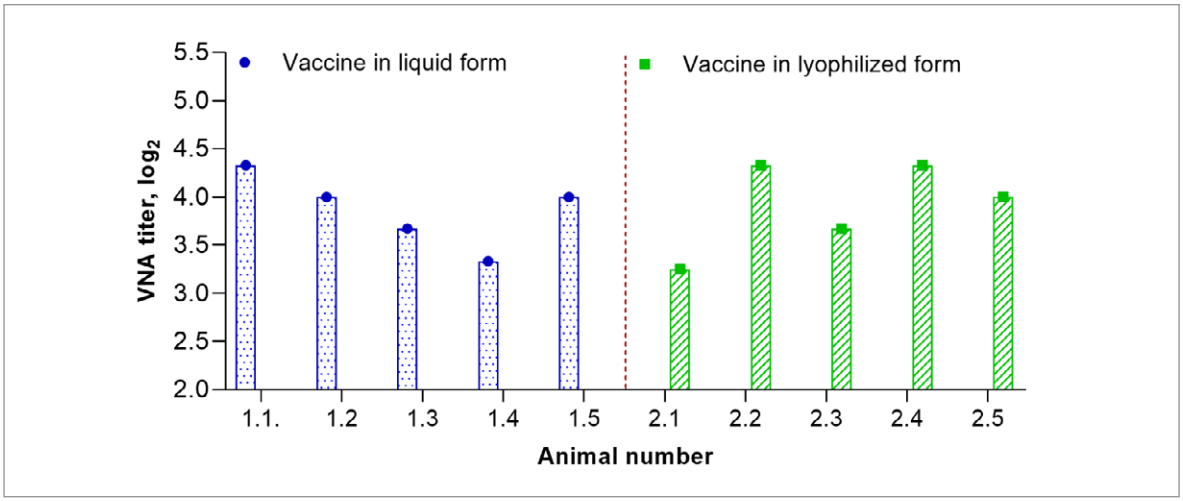

Fig. Effectiveness of oral vaccination of dogs with liquid and freeze-dried vaccines

Table
Challenge of vaccinated dogs with virulent CVS strain of rabies virus

Animal Group	Animal Number	Infection dose, MLD_{50}	Result
Group One (experimental)	1.1	100	–
	1.2	100	–
	1.3	100	–
	1.4	1,000	–
	1.5	1,000	–
Group Two (experimental)	2.1	100	–
	2.2	100	–
	2.3	100	+
	2.4	1,000	–
	2.5	1,000	–

"+" – the animal is affected;
"–" – the animal is not affected.

vaccination, blood samples were taken from all the dogs, the obtained sera were tested in VNT [23] to determine the titers of virus neutralizing antibodies (VNA).

Challenge. In order to assess anti-rabies immunity, on Day 21 post vaccination all the animals were intracerebrally infected with virulent rabies virus strain CVS at a dose of 100 and 1,000 MLD_{50} and clinically observed for 21 days.

Diffusion precipitation reaction (DPR). DPR was carried out according to GOST 26075-2013[3].

Statistical data processing. Statistical analysis was performed using GraphPad Prism Version 8.0.1. Two-way ANOVAs test was used to analyse serological test results after vaccination with both vaccines, as well as the difference between the groups after the challenge. The value of $P \leq 0.05$ was considered statistically significant. The difference in the vaccination effectiveness between the groups was assessed by One-Sided Fisher's Exact Test for two proportions at the Alpha significance level < 0.05.

RESULTS AND DISCUSSION

Observation of the vaccinated animals revealed that dogs remained healthy for 21 days after immunization, no changes in behavior or rabies clinical symptoms were recorded, thus, suggesting that the oral vaccines used in the experiment were safe.

Postvaccinal immunity was assessed by the level of rabies VNA in the vaccinated animals. The experiment results are shown in the figure.

It was found that dogs from Group One vaccinated with the liquid vaccine had VNA titers ranging from 3.33 to 4.33 log_2. VNA titre in Group Two immunized with freeze-dried virus-containing suspension was in the range from 3.25 to 4.33 log_2. Despite different forms of the oral

[3] GOST 26075-2013. Animals. Methods of Laboratory Diagnostic of Rabies. Moscow: Standartinform; 2014. 10 p. Available at: https://base.garant.ru/70995746.

vaccine used in the experiment, the maximum level of VNA in both groups of the vaccinated animals was 4.33 $\log_2$, while there was no significant difference between VNA titers in Group One and Group Two ($P > 0.05$).

It was demonstrated that the VNA developed in dogs of both groups immunized with different forms of the oral vaccine protected against infection with virulent rabies virus strain CVS. The experiment results are given in the table.

After intracerebral infection, all animals of Group One remained clinically healthy for 21 days, regardless of the infection dose. In Group Two, one dog (No. 2.3) died on Day 3 after infection, the rest of the animals were clinically healthy throughout the whole observation period, regardless of the dose of infection. As DPR showed no specific rabies virus antigen was detected in the brain samples taken from the dead dog. All animals of the control group died within 5–8 days demonstrating clinical signs of paralytic form of rabies. Specificity of the disease and the death of dogs were confirmed in the DPR.

The results obtained show that both forms of the oral rabies vaccines are effective.

CONCLUSION

Analyzing the data obtained, it can be concluded that both tested rabies vaccines, produced on the basis of rabies virus strain VRC-RZ2 and used orally for dog vaccination, induce virus neutralizing antibody response resulting in 100% protection against intracerebral infection with virulent rabies virus strain CVS. Taking into account that the freeze-dried oral vaccine is more stable at low temperatures, it can be used in various geographical zones of Kazakhstan.

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Comparative characterization of *Leporipoxvirus* members' reproduction in continuous cell culture

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SUMMARY

Examination of the virus-cell interactions is of both scientific and practical importance. Our study was aimed at comparative characterization of rabbit myxoma virus and Shope fibroma virus biological properties that manifested during the virus reproduction in RK-13/2-03 clonal continuous rabbit kidney cell culture. It was demonstrated that the viruses varied in infection development and cytopathic effect duration in RK-13/2-03 cell culture. Apparent lesions in cell monolayers infected by myxoma virus and fibroma virus at similar multiplicity of infection and cultivation temperature were observed on day 2 and day 3 of cultivation, respectively, as well as maximum cell lesions with evident degeneration were observed on day 3 and day 6 of cultivation, respectively. Myxoma virus was accumulated at titre of 6.25–6.50 lg $TCID_{50}$/0,2 cm^3, and Shope fibroma virusa was accumulated at titre of 5.50–5.75 lg $TCID_{50}$/0.2 cm^3. Shope fibroma virus demonstrated such infectivity during three passages and myxoma virus demonstrated such infectivity during twenty passages. Prepared cultures were identified as myxoma virus and Shope fibroma virus with molecular genetic analysis. Tests of the viruses for their antigenic relatedness showed that antibodies against myxoma virus were able to neutralize Shope fibroma virus also. NT titres of antibodies against both viruses were similar (1:8). RK-13/2-03 cell culture was found to be highly permissive to Shope fibroma virus that had been isolated from the diseased rabbit and not been an attenuated variant.

Keywords: continuous cell culture, myxoma virus, Shope fibroma virus, virus-specific cytopathic effect

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Сравнительная характеристика репродукции вирусов рода *Leporipoxvirus* в перевиваемой культуре клеток

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РЕЗЮМЕ

Изучение характера взаимодействия вируса с клеткой имеет как научное, так и практическое значение. Целью настоящих исследований явилась сравнительная оценка проявления ряда биологических свойств вирусов миксомы и фибромы Шоупа кроликов при репродукции в клональной перевиваемой линии клеток почки кролика RK-13/2-03. Показано, что развитие инфекции и длительность проявления цитопатического действия в культуре клеток RK-13/2-03 изучаемых вирусов различны. Видимые поражения клеточного монослоя при одинаковой множественности заражения и температуре

культивирования для вируса миксомы наблюдали на 2-е сут, для вируса фибромы – на 3-и сут, максимальное поражение клеток с выраженной дегенерацией отмечали на 3-и и 6-е сут культивирования соответственно. Вирус миксомы накапливался в титре 6,25–6,50 lg $ТЦД_{50}$/0,2 $см^3$, а вирус фибромы Шоупа – в титре 5,50–5,75 lg $ТЦД_{50}$/0,2 $см^3$. Данную инфекционную активность регистрировали у вируса фибромы Шоупа на протяжении трех, а вируса миксомы – двадцати пассажных уровней. Молекулярно-генетический анализ подтвердил, что полученные культуральные материалы идентифицируются как вирусы миксомы кроликов и фибромы Шоупа. При определении антигенного родства было установлено, что антитела, полученные к вирусу миксомы, способны нейтрализовать и вирус фибромы Шоупа. Титр антител в реакции нейтрализации обоих вирусов был идентичен и составил 1:8. Установлен высокий уровень пермиссивности культуры клеток RK-13/2-03 к вирусу фибромы Шоупа, изолированному от больного кролика и не являющемуся аттенуированным вариантом вируса.

Ключевые слова: перевиваемая культура клеток, вирус миксомы, вирус фибромы Шоупа, цитопатическое действие вируса

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INTRODUCTION

Continuous cell lines used as a cell substrate for virus and antigen accumulation for antiviral vaccine development have significantly contributed to biotechnological process effectiveness and stability. Use of continuous cell lines has contributed to significant decrease in risks of endogenous contamination that could be associated with used primary cell culture and to solving of some ethical problems associated with donor tissue collection from mammals and in general enhancement of target product biosafety.

Possibility of continuous cell line use for cultivation depends mainly on permissiveness of the selected cell system for the virus.

Consequently, examination of virus-cell interactions and subsequent optimization of cultivation conditions and parameters are of both scientific and practical significance.

Viruses of *Poxviridae* family infect many mammal species, propagate in target cell cytoplasm affecting both skin and internal organs and can be responsible for epidemics causing significant economic losses [1, 2]. The family is divided into two subfamilies: *Chordopoxvirinae* and *Entomopoxvirinae* [3]. The first subfamily comprises 11 virus genera that cause disease in animals (vaccinia, monkeypox [4], buffalo pox [5], sheep and goat pox [6], lumpy skin disease [7], fowl pox [8], rabbit myxomatosis and fibromatosis [9, 10], squirrel pox [11], etc.), as well as in humans (smallpox). The second subfamily comprises 3 virus genera circulating in insects. There are genera in *Chordopoxvirinae* that have limited host range including viruses of *Leporipoxvirus* genus causing disease only in rabbits, hares and squirrels.

Rabbit fibromatosis (Shope fibroma) is a viral disease of domestic and wild rabbits characterized with confined nodules and diffuse cirrhosis (fibromas) under skin and mucosa, the main pathognomonic feature of disease.

Rabbit myxomatosis is an infectious acute highly contagious viral disease characterized with serous-purulent conjunctivitis, edematous-tremelloid cell infiltration on head and perineum, skin tumors.

Both diseases are caused by DNA viruses. These viruses do not significantly distinct from vaccinia virus in their morphological features. Mature myxoma virus is 250–370 nm in size, and mature Shope fibroma virus is 125–240 nm in size. Myxoma virus virions are of brick-like form with rounded corners, have villous outer surface, dumb bell-shaped nucleotide and double-stranded DNA [12].

Mature Shope fibroma virus virion is of brick-like form with rounded edges. Fibroma and myxoma viruses are related in antigenic and immunogenic properties and therefore rabbits convalescent from fibromatosis are resistant to myxomatosis agent [13].

Both viruses replicate in chicken embryonated eggs but cause different lesions. Myxoma virus cultivation on chorioallantoic membrane results in formation of characteristic foci (pustules) and subsequent embryo death, whereas fibroma viruses induces mild changes and embryo remains alive. Myxoma virus causes apparent cytopathic effect characterized with cell rounding and cell layer degeneration in primary and continuous newborn rabbit kidney cell cultures. Fibroma virus reproduction in primary and continuous cell culture induces mild cytopathic effect [12].

Poxvirus reproduction in cell culture is characterized by its replication in the cytoplasm and release of the mature virions from the cell with its destruction or without cell membrane disruption (exocytosis). The induced cytopathic effect is characterized with pyknosis, cell shrinkage and destruction. However, members of the said virus

family have their own specific manifestations during their *in vitro* interactions with cells. Hinze and Walker detected mild cellucidal effect caused by Shope fibroma virus cultivated in rabbit kidney cells [14].

There are limited data on cultivation of *Leporipoxvirus* family viruses in Russian literature.

Considering the abovementioned, our study was aimed at comparative characterization of myxoma and Shope fibroma virus biological properties manifested during their reproduction in RK-13/2-03 clonal continuous rabbit kidney cell culture as a basis for improvement of the technology for production of vaccines against the diseases caused by these pathogens.

MATERIALS AND METHODS

A clone of continuous RK-13/2-03 rabbit kidney cell subline (Cat. No. 36.2) obtained from the FRCVM Cell Culture Collection [15]; rabbit-pathogenic Shope fibroma virus isolated from pathological materials (infectivity at 5th passage in rabbits – 4.11 lg ID_{50}/ml); rabbit myxoma virus (B-82 vaccine strain) with infectivity of 6.5 lg $TCID_{50}$/cm^3 were used. The virus strains were obtained from the Governmental Collection of Microorganisms Causing Dangerous, Highly Dangerous Including Zooanthroponic and Foreign Animal Diseases (CKP Reg. No. 441429, http://ckp-rf.ru/ckp/441429/).

Specific rabbit serum against myxoma virus (NT titre – 1:8) was used.

The following nutrient media were used: liquid Eagle's DMEM medium supplemented with alanyl-glutamine and glucose, 4.5 g/l, and Dulbecco solution (NPP PanEco, Russia); Eagle's MEM (Sigma, USA); fetal bovine serum (Biological Ind., Israel).

RK-13/2-03 rabbit kidney cell culture was cultivated in culture flasks (25 cm^2 growth area) and 96-well plates (Corning, USA).

RK-13 cell subline was cloned to increase genetic homogeneity level using end-point dilution method in 96-well plastic plates [16]. Prepared clones were cultivated in Eagle's MEM containing 20% of fetal bovine sera and 50% of conditioned medium with pefloxacin (antibiotic) (20 μg/ml) for 10–14 days. The most promising RK-13/2-03 clone was selected for further cultivation out of 42 line clones based on their growth and virus-reproducing property assessment results. Karyologic analysis at 18th passage showed that cells with 56 chromosomes corresponded to the modal number that was 22%. Cytomorphological examinations showed that clonal culture monolayer consisted of epithelial-like cells of polygonal shape. The monolayer formed within 48–72 hours at the seeding density of 100 ths cells/ml. The prepared clonal RK-13/2-03 cell subline retained its original growth properties and morphological characteristics through 50 passages (observation period).

Shope fibroma and myxoma (B-82 strain) viruses were cultivated in 2-day-old RK-13/2-03 cell culture grown in DMEM supplemented with 10% fetal bovine serum. Before inoculation the growth medium was removed from the flasks, the cell monolayer was washed twice with Dulbecco solution for removing remained serum and then the viruses were added at the multiplicity of infection of 0.002–0.003 $TCID_{50}$/cell. The virus was left for adsorption at (33.0 ± 0.5) °C for 60 min. Then, maintenance Eagle's DMEM supplemented with 2% fetal bovine serum was added to the flasks. Inoculated cell culture was incubated at (33.0 ± 0.5) °C up to the evident virus-induced cytopathic effect (CPE). The cell monolayer was observed for the virus-induced lesions by examination of the culture flasks under Olympus inverted microscope (Olympus, Japan).

Shope fibroma and myxoma virus infectivity was determined by conventional method of titration in RK-13/2-03 cell culture. For this purpose, RK-13/2-03 cells were seeded in 96-well plates at concentration of 150 ths cells/cm^3. Two days later, the cells were inoculated with 10-fold dilutions (10^{-1}–10^{-7}) of Shope fibroma and myxoma viruses upon confluent monolayer formation and then were incubated at temperature of (33.0 ± 0.5) °C in 5% CO_2 atmosphere and at 95%-relative humidity. The observation period was 10 days. Titration results were assessed based on the virus-induced CPE, the titre was calculated using Reed and Muench method and expressed as lg $TCID_{50}$/0.2 cm^3.

Virus DNAs were extracted with QIAamp DNA Mini kit (Qiagen, Germany) according the manufacturer instruction.

Primers proposed by Y. Li et al. [17] and S. Albini et al. [18] were used for detection of Shope fibroma and myxoma virus genomes.

Polymerase chain reaction (PCR) products were purified with Cleanup Standard kit (ZAO Evrogen, Russia) with subsequent Sanger direct sequencing using Applied Biosystem 3130 XL DNA Analyser (Applied Biosystems, USA) and reagents of BigDye™ Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, USA).

Conventional neutralization test was used for demonstration antigenic relatedness between myxoma and Shope fibroma virus. RK-13/02-03 cell suspension (at concentration of 100 ths cells/cm^3) was added to 96-well plates and the plates were incubated at temperature of (37.0 ± 0.5) °C in 5% CO_2 atmosphere and at 95%-relative humidity for 48 hours. Normal and myxoma virus-specific rabbit sera were titrated in duplicate (1:2–1:64) in two plates; then, 1,000 doses of myxoma virus were added to the first plate and 1,000 doses of Shope fibroma virus were added to the second plate. Serum-virus mixture was kept at temperature of (33.0 ± 0.5) °C in 5% CO_2 atmosphere and at 95% relative humidity and transferred to the plates with cell culture and then incubated under the abovementioned conditions for 10 days. Results were recoded starting with day 3.

RESULTS AND DISCUSSION

Apparent manifestations of the viruses' reproduction were observed on day 2 (Fig. A) and day 3 (Fig. B) of incubation of myxoma virus (B-82 strain) and Shope fibroma virus, respectively, in RK-13/2-03 cell culture. Lesions in the culture caused by viruses at the beginning of the infection were characterized with rounded cell accumulation and small bundle cluster formation. Characteristic small disruptions of the cell layer were observed in the culture infected with myxoma virus. Changes in the infected monolayer: cell layer destruction with the formation of a "grid" of elongated cellular elements surrounded by rounded cells with sharply contoured thick walls, became more apparent on day 3 of this virus cultivation (Fig. C).

Fig. Dynamics and nature of CPE caused by myxoma viruses and Shope fibroma virus in RK-13/2-03 cell culture (magnification 100×). Myxoma virus manifestations on day 2 (A), Shope fibroma virus manifestations on day 3 (B), myxoma virus-associated lesions on day 3 (C), fibroma virus-associated lesions on day 4 (D), fibroma virus-associated lesions on day 5 (E), intact RK-13/2-03 cell (F)

Progressive destructive changes were observed in cell monolayer infected with fibroma virus on day 4 of incubation (Fig. D); maximum cell lesions with evident degeneration were observed on day 5–6 of cultivation (Fig. E). No such changes were observed in intact RK-13/2-03 cell culture (control) (Fig. F).

Thus, dynamics of myxoma virus and Shope fibroma virus –induced CPE was different in RK-13/2-03 cell culture and the CPE became apparent on day 3 and 6, respectively. The nature of cell monolayer lesions was identical throughout the whole cultivation cycle.

Some differences were found when viruses' accumulation during cultivation. Myxoma virus infectivity reached 6.25–6.50 lg $TCID_{50}$/0.2 cm^3 during cultivation period (3 days), whereas Shope fibroma virus titre was 5.50–5.75 lg $TCID_{50}$/0.2 cm^3 on day 6 of cultivation. Shope fibroma virus and myxoma virus demonstrated such infectivity levels during three and twenty passages, respectively.

Poxvirus amplicons were produced with PCR and nucleotide sequencing was carried for their identification. Resulting sequences were compared with gene sequences deposited in GenBank database using Blast software (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Molecular genetic analysis confirmed that the resulting virus-containing materials were myxoma and Shope fibroma viruses.

Neutralization tests of the viruses for their antigenic relatedness showed that antibodies against myxoma virus were also able to neutralize Shope fibroma virus. Antibody titres against myxoma and Shope fibroma viruses were identical and equaled to 1:8. It was indicative of antigenic relationship between myxoma and Shope fibroma viruses.

Significant differences in dynamics of CPE caused by Shope fibroma and myxoma viruses in RK-13/2-03 cell culture were found. Apparent cell monolayer lesions induced by myxoma virus and Shope fibroma virus at similar multiplicity of infection and cultivation temperature were observed on day 2 and day 3, respectively, that was consistent to the findings obtained during reproduction of these viruses in primary cell cultures [12].

Cytomorphological examinations showed that cell culture lesions induced by the studied viruses were characteristic of *Poxviridae* family members; rounded cells and their agglomerations in the form of pox plaques were the main CPE manifestations [19].

The above-mentioned CPE differed from the CPE induced by Mix-98 strain of myxoma virus characterized by rounded cell pulling to the lesion centre with spider-like bundle formation [20].

These two antigenically related viruses cultured under similar conditions differed in their infectivity. Myxoma virus accumulated at higher titres since its B-82 vaccine strain had been adapted to the said cell culture. However, Shope fibroma virus isolated from organ lesions without preliminary adaptation to RK-13/2-03 cells also actively propagated in the cells as compared to other viruses of *Poxviridae* family. For example, lumpy skin disease virus required longer period of adaptation to continuous cell cultures. Characteristic lesions in RK-13/2-03 cell monolayer were reported starting with the 3rd passage, no visible changes were observed in 1st-passage cell culture, minor changes were observed in 2nd-passage cell culture [21].

Studies of these two viruses showed no distinct cultivation peculiarities and confirmed their antigenic relatedness. Sequencing of culture virus-containing materials showed their relevance to the taxonomic status.

CONCLUSION

Comparative characterization of myxoma virus and Shope fibroma virus reproduction in continuous rabbit kidney cell line revealed differences in cytopathic effect dynamics. The viruses induced identical lesions in RK-13/2-03 cell culture characterized by rounding of epithelium-like monolayer cells followed by their blotch-like agglomeration. Increasing destructive processes result in cell layer destruction, formation of grid-like structures surrounded by rounded cells.

It was found that myxoma virus being a culture vaccine strain (B-82) had a higher infectivity.

Neutralization test demonstrated that myxoma virus and Shope fibroma virus were antigenically related and belonged to *Leporipoxvirus* genus.

RK-13/2-03 cell culture was found to be highly permissive to virulent Shope fibroma virus isolate recovered from the diseased rabbit.

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Echinococcosis of dogs in the North Caucasian Subjects (infrastructural, epizootological and sanitary-hygienic analysis)

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SUMMARY

Based on the official statistics the situation of parasitic diseases in the Russian Federation is still quite unfavorable. The issues of soil contamination with *Echinococcus granulosus* eggs are understudied that's why the objective of the research was to study canine echinococcosis which poses a threat to animal and human disease freedom in the North Caucasian countries. Helminthological tests of the guardian dog feces were performed in seven North Caucasian Subjects: the Stavropol Krai, the Karachay-Cherkessia Republic, the Kabardino-Balkaria Republic, Republic of North Ossetia –Alania, Republic of Ingushetia, the Chechen Republic, and the Republic of Dagestan. As a result, it was determined that the average frequency index of echinococcosis occurrence was 85.07%. The moderate fecal egg count in 1,400 samples was 22.73 ± 1.49 eggs per 10 grams of dog feces. The data obtained are indicative of the disease unfavourable situation in the Subjects and the zoonosis spread at the regional level. Helminthological tests of 14,000 soil samples from near-village pastures for contamination with tapeworm eggs, including *Echinococcus granulosus*, showed that the invasion rate in the Stavropol Krai was 65.80%, in Karachay-Cherkessia republic – 79.00%, in Kabardino-Balkaria – 82.60%, in North Ossetia – Alania – 74.65%, in Ingushetia – 88.20%, in Chechnya – 83.75%, in Dagestan – 79.85%. The results obtained testify to the high level of soil contamination with the infective eggs. It was demonstrated that there is a relationship between the distribution of viable *Echinococcus granulosus* eggs in pasture soils and ecological characteristics of the Subject: the largest number of viable *Echinococcus granulosus* eggs was observed in submountain areas, fewer eggs were observed in flatlands, and the least number of eggs – in the mountain areas. The number of eggs detected in the soil samples from pastures is indicative of the disease persistence in humans and animals. Results of the helminthological tests of 7,500 soil samples from 119 cattle-driving routes of the North Caucasus demonstrate 100% contamination with parasitic agents which poses a threat of epidemiological and epizootological situation of echinococcosis in the Subject.

Keywords: North Caucasus, guardian dogs, cestode, *Echinococcus granulosus* species, examination, feces, eggs, distribution, pollution, pastures, soil

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Эхинококкоз собак в субъектах Северного Кавказа (инфраструктурный, эпизоотологический и санитарно-гигиенический анализ)

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РЕЗЮМЕ

Согласно данным официальной статистики, в Российской Федерации ситуация по паразитарным болезням остается неблагополучной. Вопросы санитарного загрязнения почв разных объектов яйцами *Echinococcus granulosus* остаются недостаточно изученными, поэтому целью исследования являлось изучение эхинококкоза собак как угрозы эпизоотическому, эпидемиологическому и санитарно-гигиеническому благополучию субъектов Северного Кавказа. Гельминтологические исследования проб фецес приотарных собак провели в семи регионах Северного Кавказа: Ставропольском крае, Карачаево-Черкесской Республике, Кабардино-Балкарской Республике, Республике Северная Осетия – Алания, Республике Ингушетия, Чеченской Республике, Республике Дагестан. В результате установлено, что средний индекс встречаемости инвазии эхинококкоза составил 85,07%. В 1400 пробах средний индекс обилия яиц *Echinococcus granulosus* был равен 22,73 ± 1,49 экз. в 10 г фецес собак. Полученные данные свидетельствуют о санитарном неблагополучии субъектов по данной инвазии и широком распространении зооноза в региональном масштабе. Санитарно-гельминтологические исследования 14 000 проб почвы присельских пастбищ на обсемененность яйцами тениат, в т. ч. *Echinococcus granulosus*, показали, что в Ставропольском крае инвазированность составила 65,80%, в Карачаево-Черкесии экстенсивный показатель инвазии почв составил 79,00%, в Кабардино-Балкарии – 82,60%, в Северной Осетии – Алании – 74,65%, в Ингушетии – 88,20%, в Чечне – 83,75%, в Дагестане – 79,85%. Представленные результаты указывают на высокий уровень контаминации почв региона инвазионными элементами. Показано, что обсемененность почв пастбищных угодий жизнеспособными яйцами *Echinococcus granulosus* находилась в прямой зависимости от экологических особенностей региона: наибольшее количество жизнеспособных яиц *Echinococcus granulosus* содержалось в почвах предгорной зоны, меньшее – на равнинной территории и минимальное – в горной зоне. При этом установленного количества яиц в пробах почвы пастбищ достаточно для поддержания стойкого неблагополучия субъектов по эхинококкозу животных и человека. Результаты санитарно-гельминтологической экспертизы 7500 проб почв 119 скотопрогонных трасс субъектов Северного Кавказа свидетельствуют о 100%-й обсемененности паразитарными агентами, что создает угрозу осложнения эпидемиологической и эпизоотической обстановки по эхинококкозу на территории региона.

Ключевые слова: Северный Кавказ, приотарная собака, цестода, вид *Echinococcus granulosus*, экспертиза, фецес, яйца, обсемененность, загрязнение, пастбища, почва

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INTRODUCTION

Multiple investigations showed that *Echinococcus granulosus* (*E. granulosus*) is a dangerous parasite for more than 100 animal species and humans. This helminthic species poses epidemiological risk for human health in the Russian Federation [1–12].

In accordance with the OIE and FAO the average prevalence of echinococcosis in domestic and wild carnivores in South Africa was 86.5% [13–16], in Southeast Asia – 75.9% [17, 18], in Latin America – 68.3% [19], and in North America – 62.8% [20].

Based on the OIE and FAO data echinococcosis in domestic and wild ruminants in Africa occurs in the form of epizootics, it's prevalence is 42.8%, in more than 40 of them prevalence is 58.5% [14, 21]. In Southeast Asia echinococcosis prevalence in ruminants is 39.6% [18], in Latin America – 34.5% [22], in North America – 28.4% [20, 23–25].

According to the World Health Organization (WHO) echinococcosis is among diseases of parasitic etiology posing global epidemiological risk and its prevalence is 300–1,250 cases per 100 thousand people [4].

In the Russian Federation echinococcosis of sheep and cattle is a major problem for many livestock sectors and one of the main reasons for decreased productivity in more than 500 breeds and lineages of productive animals and it 2–3.5 times exceeds the similar morbidity parameters in the European countries [26].

In the Russian Federation as well as in the entire world the contamination of soil at different infrastructure facilities with *E. granulosus* eggs is still quite understudied [2, 27].

The research was aimed at studies of echinococcosis being the threat to welfare of the North Caucasian Subjects in terms of animal and human diseases as well as compliance with sanitary and hygienic requirements.

MATERIALS AND METHODS

In seven North-Caucasian Subjects (Stavropol Krai, Karachay-Cherkess Republic, Kabardino-Balkarian Republic, Republic of North Ossetia – Alania, Republic of Ingushetia, Chechen Republic, and Republic of Dagestan) 1,400 feces samples collected from 1,400 guardian dogs were tested for *E. granulosus* epizootic activity using life-time diagnosis methods (coproscopy, helminthoscopic methods, direct smear method, Demidov and Fülleborn methods).

When carrying out a sanitary and helminthological examination of the soil for taenia egg contamination, including *E. granulosus*, 14,000 soil samples from pastures and 7,500 soil samples from 119 livestock routes were tested using certified parasitological ovoscopic methods.

Statistical processing of the material was carried out using Biometrics computer software.

RESULTS AND DISCUSSION

Helminthological tests of samples collected from guardian dog feces for echinococcosis with subsequent determination of the *E. granulosus* prevalence and abundance were performed under experimental conditions in seven North Caucasian Subjects to assess the risks for human and animal health (Table 1).

As a result, it was determined that the average prevalence of canine echinococcosis was 85.07%, which is indicative of the wide spread of the zoonotic parasite infection at the regional level.

The average index of *E. granulosus* abundance determined during tests of 1,400 feces samples was 22.73 ± 1.49 eggs in 10 g of dog feces which indicates that the Subjects are infected with the disease.

The results of examining near-village soil for contamination with taenia eggs (%), including *E. granulosus*, in the North Caucasus based on the feces sample tests are demonstrated in Table 2.

It was found that in the Stavropol Krai the pasture soils were contaminated in 65.80% of cases, in the Karachay-Cherkess Republic – in 79.00%, in the Kabardino-Balkarian Republic – in 82.60%, in the Republic of North Ossetia – Alania – in 74.65%, in the Republic of Ingushetia – in 88.20%, in the Chechen Republic – in 83.75%, and in the Republic of Dagestan – in 79.85% of cases. Thus, the prevalence of the echinococcosis agent eggs in soil samples of this category was 79.12%, which indicates a high level of contamination of the region's soils with invasive elements.

The tests of 14,000 soil samples from near-village pastures demonstrated that the abundance index of

Table 1
The results of helminthological tests of guardian dog feces samples for echinococcosis caused by the cestode *E. granulosus* in the North Caucasian Subjects (according to Ova and Parasite Test of feces samples, %)

North Caucasian Subjects	Amount of dogs tested	Amount of dogs infested with taenia eggs, including *E. granulosus*	Prevalence, %	Mean abundance index for *E. granulosus* eggs in 10 g of dog feces
Stavropol Krai	200	155	77.50	18.76 ± 1.20
Karachay-Cherkess Republic	200	170	85.00	23.40 ± 1.50
Kabardino-Balkarian Republic	200	179	89.50	26.82 ± 1.70
Republic of North Ossetia – Alania	200	164	82.00	20.79 ± 1.40
Republic of Ingushetia	200	182	91.00	28.65 ± 1.90
Chechen Republic	200	169	84.50	21.21 ± 1.30
Republic of Dagestan	200	172	86.00	19.50 ± 1.40
Total	1,400	1,191	85.07	22.73 ± 1.49

Table 2
The results of the sanitary and helminthological examination of the pasture soils for contamination with taenia eggs, including *E. granulosus*, in the North Caucasian Subjects

North Caucasian Subjects	Amount of soil samples tested	Amount of soil samples infested with taenia eggs, including *E. granulosus*	Prevalence of taenia eggs, including *E. granulosus*, %	Average abundance index for *E. granulosus* eggs in 10 g of soil samples
Stavropol Krai	2,000	1,316	65.80	15.72 ± 0.90
Karachay-Cherkess Republic	2,000	1,580	79.00	20.40 ± 1.20
Kabardino-Balkarian Republic	2,000	1,652	82.60	23.81 ± 1.40
Republic of North Ossetia – Alania	2,000	1,493	74.65	16.78 ± 1.10
Republic of Ingushetia	2,000	1,764	88.20	24.60 ± 1.50
Chechen Republic	2,000	1,675	83.75	19.20 ± 1.20
Republic of Dagestan	2,000	1,597	79.85	17.48 ± 1.10
Total	14,000	11,077	79.12	19.71 ± 1.20

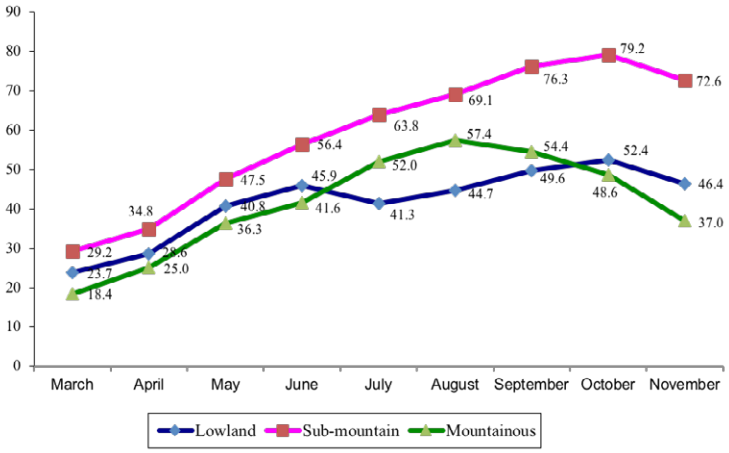

Fig. Average proportion of pasture soil samples where viable E. granulosus eggs were detected, in the warm period of 2020 in the Karachay-Cherkess Republic climatic zones, %

E. granulosus eggs was 19.71 ± 1.20 eggs per 10 g sample, which is indicative of epidemiologically hazardous contamination.

Thus, the results of helminthological tests of dog feces samples and sanitary and helminthological examination of soils showed that all seven North Caucasian Subjects (Stavropol Krai, Karachay-Cherkess Republic, Kabardino-Balkarian Republic, Republic of North Ossetia – Alania, Republic of Ingushetia, Chechen Republic and Republic Dagestan) have the status of echinococcosis infection. The reason for this is the poor implementation of anti-epizootic measures addressing this invasion and non-compliance with the dog deworming schedule.

Tests carried out during the growing season of 2020 in the lowland, sub-mountain, and mountainous areas of the Karachay-Cherkess Republic showed that the quantitative content of viable *E. granulosus* eggs in soil samples of all pasture lands directly correlated with the ecological characteristics of the region, where the determining factors are the effective temperature sum and humidity. The results are demonstrated in Figure.

So, in the lowland natural and climatic conditions in the area under study the share of samples with viable eggs of the parasite in the soil in June was 45.9% of the number of samples tested, in July – 41.3%, in October – 52.4% and at the end of November – 46.4%. In the sub-mountain area, this parameter in March was equal to 29.2%, in October – 79.2%, and in the third decade of November – 72.6%. In the mountainous area, the number of viable eggs of *E. granulosus* in soil samples from pasture lands of the Subject from March to August gradually increased from 18.4% to 57.4%, and by the end of November decreased to 37.0%.

Thus, when comparing samples from all pastures, the largest number of viable *E. granulosus* eggs was in the soils of the sub-mountain, the smallest number – in the lowland, and the minimum – in the mountainous area. At the same time, the detected amount of viable *E. granulosus* eggs in the pasture soil samples is sufficient for maintaining echinococcosis persistence in animals and humans in the region.

At the next stage of the research, a sanitary and helminthological examination of 7,500 soil samples from 119 livestock routes in six North Caucasian Subjects was carried out for the detection of taenia eggs, including *E. granulosus* (Table 3).

The results of the sanitary and helminthological examination indicate a high level of contamination of the soils from livestock routes by invasive elements of the parasite. All tested soil samples (100%) collected from the livestock routes were contaminated with viable eggs of *E. granulosus,* which may become one of the main threats to the sanitary-epidemiological and epizootic situation in six North Caucasian Subjects.

CONCLUSION

As shown by the results of the sanitary and helminthological examination of feces samples of guardian dogs in the North Caucasian Subjects (Stavropol Krai, Karachay-Cherkess Republic, Kabardino-Balkarian Republic, Republic of North Ossetia – Alania, Republic of Ingushetia, Chechen Republic, Republic of Dagestan), the average prevalence of echinococcosis invasion amounted to 85.07%, which indicates a wide distribution of this zoonosis in the region. In 1,400 feces samples, the abundance index of *E. granulosus* eggs was 22.73 ± 1.49 eggs in 10 g canine feces.

Sanitary and parasitological studies of the soils of the near-village pastures in the Stavropol Krai demonstrated that 65.80% of samples were contaminated with taenia

Table 3
The results of the sanitary and helminthological examination of the cattle-driving routes for contamination with taenia eggs, including *E. granulosus*, in the North Caucasian Subjects

North Caucasian Subjects	Cattle-driving routes under testing	Amount of tested soil samples	Amount of soil samples contaminated with *E. granulosus*	*E. granulosus* prevalence, %
Karachay-Cherkess Republic	18	900	900	100
Kabardino-Balkarian Republic	22	1,230	1,230	100
Republic of North Ossetia – Alania	15	700	700	100
Republic of Ingushetia	10	500	500	100
Chechen Republic	13	670	670	100
Republic of Dagestan	41	3,500	3,500	100
Total	119	7,500	7,500	100

eggs, including *E. granulosus*, in the Karachay-Cherkess Republic the extensive soil invasion rate was 79.00%, in the Kabardino-Balkarian Republic – 82.60%, in the Republic of North Ossetia – Alania – 74.65%, in the Republic of Ingushetia – 88.20%, in the Chechen Republic – 83.75%, in the Republic of Dagestan – 79.85%. Thus, the average prevalence of the echinococcosis causative agent eggs in soil samples of this category was 79.12%, which indicates a high level of soil contamination with cestode eggs. When testing 14,000 soil samples, the abundance index of *E. granulosus* eggs was 19.71 ± 1.20 in 10 g sample, which is evident of dangerous contamination of pasture soils in the North Caucasus.

Studies carried out during the growing season of 2020 under the conditions of different climatic zones of the Karachay-Cherkess Republic showed that the largest number of viable *E. granulosus* eggs was in the sub-mountain area, less – in the lowland and the minimum – in the mountainous area. At the same time, the detected amount of viable *E. granulosus* eggs in the pasture soil samples is sufficient to maintain the persistent infection in the entire region.

The results of the sanitary and helminthological examination of the soils from the cattle driving routes of the North Caucasian Subjects indicate a 100% contamination with taenia eggs, including *E. granulosus*, which poses a threat of worse epidemiological and epizootic situation for echinococcosis in the region.

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Coagulase gene and *agr* complex polymorphism-based genotyping of *Staphylococcus aureus* isolated from lower primates

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SUMMARY

Staphylococcus aureus is a pathogenic microorganism causing a great number of diseases in humans and animals. Many researches on genotyping *Staphylococcus aureus* isolated from humans and mastitis affected cowsare performed, but no foreign reports on typing of *Staphylococcus aureus* detected in monkeys have been found. Staphylococcus-induced infections are however widely spread in primates. The paper demonstrates results of molecular and genetic examination of *Staphylococcus aureus* isolated from lower primates. The examination was based on typing of coagulase gene and polymorphic locus of *arg* gene that regulates expression of pathogenicity-associated genes. Structures of coagulase gene (*coa*) and polymorphic types of regulatory gene (*agr*) were studied in 145 *Staphylococcus aureus* isolates recovered from various monkey species. The studies resulted in singular coagulase gene fragments of four dimensions: 600, 750, 800 and 900 bps. Following *AluI* endonuclease restriction results *Staphylococcus aureus* was classified in seven different *coa*-types. Coagulase gene of genotype VII predominated (31.7%), genotype II was detected less frequently (9.7%). Each *Staphylococcus aureus* isolate is specified by a definite coagulase gene restriction profile; therefore, at least seven *Staphylococcus aureus* strains are currently circulating in the monkeys in the monkey facilities. Herewith, those staphylococci that bear genotype VII coagulase gene are invasive as they are isolated from various organs and pus as well as from feces and nasal cavities of the animals. Analysis of the study results demonstrated that bacteria of this species could be transmitted between different monkey species. Apart from human *Staphylococcus aureus*, in whose genome *agrI* prevails, *agrIV* prevailed in the isolates outlined in this paper (59.3%); *agrII* and *agrIII* were detected in 5.5 and 2.1% of the isolates, respectively.

Key words: monkeys, *Staphylococcus aureus*, coagulase gene, regulatory gene, *agr* groups

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Генотипирование *Staphylococcus aureus*, выделенного у низших приматов, на основе полиморфизма коагулазного гена и генов комплекса *agr*

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РЕЗЮМЕ

Золотистый стафилококк (*Staphylococcus aureus*) – патогенный микроорганизм, вызывающий большое количество заболеваний у человека и животных. Проведено много исследований по генотипированию *Staphylococcus aureus*, выделенных у людей и при маститах у коров, но данных по типированию *Staphylococcus aureus*, обнаруженного у обезьян, в зарубежной литературе не найдено. Однако инфекции стафилококковой природы у приматов распространены широко. В статье представлены результаты молекулярно-генетического изучения золотистых стафилококков, изолированных от низших обезьян, на основе типирования коагулазного гена и полиморфного локуса *agr*, являющегося регулятором экспрессии генов патогенности. Проведено

исследование структуры коагулазного гена (*coa*) и полиморфных типов регуляторного гена (*agr*) у 145 изолятов *Staphylococcus aureus*, выделенных от обезьян разных видов. Получены одиночные фрагменты коагулазного гена четырех размеров: 600, 750, 800 и 900 п. н. По результатам рестрикции эндонуклеазой *AluI* изученные золотистые стафилококки классифицированы на семь различных *coa*-типов. Наиболее часто обнаруживали VII генотип (31,7%), реже – II генотип коагулазного гена (9,7%). Для каждого изолята *Staphylococcus aureus* характерен определенный профиль рестрикции коагулазного гена, следовательно, среди обезьян питомника циркулирует как минимум семь штаммов золотистого стафилококка. При этом стафилококки, содержащие коагулазный ген VII генотипа, являются инвазивными, так как выделены из различных органов и гноя, а также фекалий и носовой полости животных. Анализ результатов исследования показал, что между разными видами обезьян возможна передача бактерий данного вида. Установлено, что, в отличие от обнаруженных у людей изолятов *Staphylococcus aureus*, в геноме которых преобладает *agrI*, у изученных в настоящей работе изолятов превалирует *agrIV* (59,3%). Группы *agrII* и *agrIII* детектированы у 5,5 и 2,1% изолятов соответственно.

Ключевые слова: обезьяны, *Staphylococcus aureus*, коагулазный ген, регуляторный ген, группы комплекса *agr*

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INTRODUCTION

Staphylococcus aureus is the cause of human and animal diseases including benign infections of the skin and soft tissues [1, 2]. Danger of the pathogen involves its capacity to cause severe infections to the extent of lethal ones (pneumonia, sepsis, etc.). This is due to the action of a large number of virulence factors produced by it, such as staphylokinase, adhesins, hemolysines, leukocidins, enterotoxins, etc. [2, 3], whose expression is regulated by aspecific system – additional regulator gene *agr* [2, 4–7]. Although this locus is a conservative one, it consists of polymorphic hypervariable fragment used for classifying *St. aureus* strains to one of four groups (polymorphic types) – *agrI*, *agrII*, *agrIII*, *agrIV* – by polymerase chain reaction (PCR) [4, 8]. Some researchers insist that *agr*-groups influence the virulence of the strains and disease progression [9].

In the late twentieth century, molecular typing methods became applicable for the research of different pathogenic microorganisms, and they allowed for the detection of epidemically significant strains. Such methods allowed detection of genetic determinants of *St. aureus* virulence and therefore it was established that this microorganism belongs to heterogenic and polymorphic species, in which pathogenicity genes are located in the chromosomal pathogenicity islands, chromosomal cassettes and prophages. In different strains, these genetic elements are present as allelic forms and specified by different degree of mobility [4]. Horizontal transfer of genes located on the mobile genetic elements results in the genetic diversity within *St. aureus*.

One of the key *St. aureus* virulence factors is coagulase – extracellular product of the strains and it stimulates prothrombin thus resulting in blood clotting. Coagulase gene *coa* is genetically variable, i.e. polymorphic [10, 11]. Coagulase typing was developed as one of the molecular tools used for identification and differentiation of *St. aureus* strains [12, 13]. Coagulase gene amplification is considered simple and accurate typing tool for *St. aureus* isolated from different sources and bearing relevant information on the genetic background of the isolates [14, 15]. This method is based on the detection of variability and polymorphism resulted from the mutations occurring on coagulase gene 3´-terminal bearing tandem arrays of 81 base pairs (bp) and changing the size of the gene [16–19]. DNA fragments associated with the coagulase gene variable region are subjected to PCR-amplification followed by restriction enzyme (endonuclease) cleavage and analysis of the different restriction fragment lengths or patterns (PCR-RFLP).The differentiation of isolates is based on the data on the number and size of such fragments. Therefore, examination of the polymorphism of such *St. aureus* virulence genes as coagulase and regulatory ones can be of diagnostic importance [12, 20]. Coagulase gene genotypes are well studied in case of *St. aureus* spp. isolated from humans [21]. Many studies were also devoted to the examination of the *coa*-gene polymorphism in *St. aureus* spp. Isolated from mastitis-affected caws and milk [1, 6, 11, 15, 22–25].These studies demonstrated that different *St. aureus* genotypes can be isolated from mastitis-affected cows not only in different geographical locations but within the same herd [6]. According to the published data, the strains can be transmitted between humans and different animals including monkeys [24]. Human strains of *St. aureus* colonize and infect monkeys, both captured and wild [26, 27]. Acquisition and loss of genes located on mobile genetic elements are considered the main factor of the microbe adaptation after transmission between the hosts [26].

No research data on coagulase gene polymorphism and allelic variants of the regulatory gene of *St. aureus* isolated from various monkey species were found in published reports.

The purpose of this work is to carry out genotyping of *St. aureus* isolates recovered from various monkey species basing on the coagulase gene polymorphism and polymorphic types of the regulatory gene.

MATERIALS AND METHODS

We studied 145 *St. aureus* isolates recovered as are result of bacteriological tests of 33 live and 100 dead monkeys of various species (*Macaca mulatta* – 51, *Macaca fascicularis* – 33, *Papio hamadryas* – 33, *Papio Anubis* – 9, *Chlorocebus sabaeus* – 3, *Macaca nemestrina* – 3, *Cebus capucinus* – 1), kept in captivity in the Adler monkey colony. Monkeys of both sexes (♀ – 69, ♂ – 64) and of different age (from day 0 to 35 years of age) were tested. Swabs from the nasal mucosa ($n = 11$), pharynx ($n = 1$), urethra ($n = 1$), wound pus ($n = 2$) were taken from living monkeys for the study. Fecal samples were collected from rectum ($n = 18$). In case of dead animals, test materials included pieces of organs and biological fluids collected during post-mortem examination (lungs – 66, liver – 11, spleen – 12, kidney – 6, lymph node – 9, uterus – 1, caecum 4, pus – 2, biological fluid – 1).

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

The material was inoculated to the salt agar with egg yolk and 5% blood agar and incubated at 37 °C for 24–48 hours. The grown staphylococcus colonies were examined morphologically (based on description of colonies, their pigmentation, Gram staining) and biochemically, taking note of lecithinase activity in salt agar with egg yolk, hemolytic activity in blood agar as well as their blood clotting activity. The species were identified using commercial test-kits 'Multimicrotests for biochemical identification of staphylococci (MMTS)' (OOO NPO Immunotex, Russia). Until further tests, the *St. aureus* isolates were stored at minus 20 °C.

DNA extraction and *agr*-typing were performed as described above [28].The coagulase gene was detected according to the procedure described in the previous paper [29]. Coagulase gene structural polymorphism was examined according to J. V. Hookey et al. [30] using *AluI* restriction endonuclease (New England BioLabs, USA) according to the following procedure: 5 µl 10× NEBuffer, 34 µl of deionized water, 10 µl of DNA, 1 µl of *AluI*. The mixture was incubated for 1 hour at 37 °C. Further analysis was based on the obtained amplicons, amount and length of the restriction fragments. *Coa*-PCR-RFLP-patterns were demonstrated as numerical code: first number (before slash) corresponds to the PCR-product length; next (after slash) lengths of restriction *AluI*-fragments are designated.

The amplification products were visualized in Tris-acetate buffer (TAE) using 2% agarose gel (Sigma, USA) stained with ethidium bromide solution (0.5 µg/ml) at 130V gradient for 50 min (electrophoresis of the restriction products occurred at 80V for 1 hour 35 min). Upon the electrophoresis completion, the results were visualized in UV transilluminator (wave length 254 nm). The size of the amplicons was determined using 100-1200 bp DNA ladder (Evrogen, Russia). There action results were UV photographed.

Table 1
Specification of coagulase gene PCR-products (amplicons) of *St. aureus* isolated from monkeys

coa-type	PCR-product size (~ number of base pairs)	Number of isolates (%)	Number of PCR-patterns
1	600	42 (29%)	1
2	750	17 (12%)	1
3	800	19 (13%)	3
4	900	67 (46%)	3

RESULTS AND DISCUSSION

Four types of amplicons were identified following the results of the coagulase gene amplification (Table 1). The table demonstrates that the majority of the isolates (46%) contained 900 bp type IV coagulase gene.

Use of *AluI* endonucleases allowed for identification of seven different coagulase gene restriction profiles (genotypes) in the tested isolates that provided from one to three restriction fragments varying size – from 80 to 750 bp (Table 2).

Table 2
Frequency of monkey's *St. aureus* coagulase gene and *agr* detection by genotypes and types

coa-gene genotype (restriction profile)	*coa*-PCR-RFLP-patterns, bp	*agr*-type (number)	Total
I	600/600	*agrI* – 6 *agrIV* – 22	28 (19.3%)
II	600/300	*agrI* – 11 *agrII* – 2 *agrIV* – 1	14 (9.7%)
III	750/750	*agrI* – 5 *agrII* – 1 *agrIV* – 11	17 (11.7%)
IV	800/400-220-80	*agrI* – 4 *agrIV* – 15	19 (13.1%)
V	900/220-180-80	*agrI* – 8 *agrII* – 2 *agrIII* – 1 *agrIV* – 7	18 (12.4%)
VI	900/450-220-80	*agrI* – 3	3 (2.1%)
VII	900/550-220-80	*agrI* – 11 *agrII* – 3 *agrIII* – 2 *agrIV* – 30	46 (31.7%)

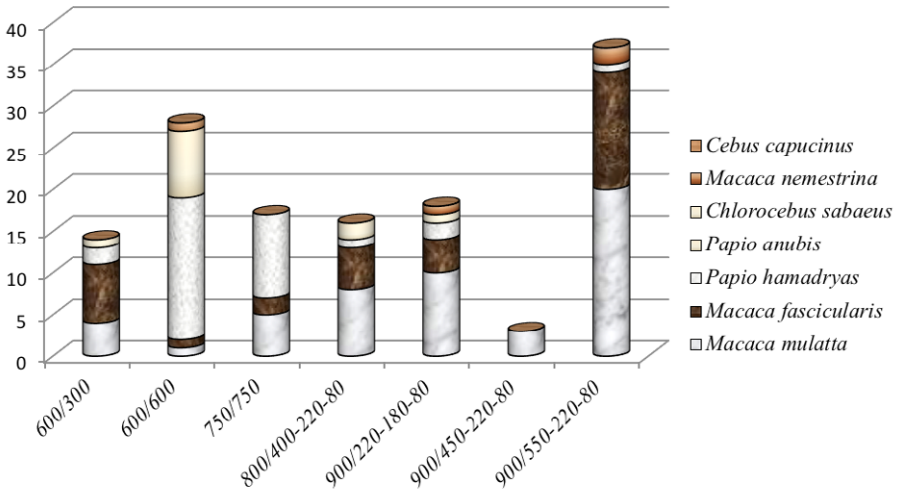

Fig. Coagulase gene restriction profiles of St. aureus isolated from different monkey species

Wherein, similar length of amplicons and specific restrictions were typical for each group. Absence of any difference in the amplicons size is indicative of the absence of the difference in the number of nucleotide repeats, while similar restriction sites indicate structural similarity of these repeats. The majority of *St. aureus* isolates belonged to type VII (31.7%); less frequently genotype VI coagulase gene was detected (2.1%).

It should be noted that the restriction profile of 600/600 bp (*coa*-gene of genotype I) was frequently observed in *St. aureus* isolated from baboons: in 25 out of 42 tested isolates (Figure). Detection of coagulase gene with such structure in two macaques and capuchin can be explained by the fact that these animals were quarantined together with baboons (*St. aureus* infected or carriers) that resulted in their infection with this isolate. It can be therefore supposed that *St. aureus* circulates between the monkeys at the quarantine facilities.

Pattern 900/550-220-80 (genotype VII) was more frequently detected in *St. aureus* isolated from macaques: in 36 out 87 tested isolates. Only three rhesus macaques demonstrated coagulase gene having 900/450-220-80 pattern (*coa*-gene of genotype VI). While each isolate is specified by its own restriction profile, the obtained data demonstrate that at least seven *St. aureus* isolates circulate in monkeys kept in Adler monkey colony.

St. aureus strain containing genotype VII *coa*-gene (900/550-220-80) is invasive because it is isolated from all organs and pus (Table 3).

On the other hand, the majority of *St. aureus* isolates detected in feces and nasal cavities of clinically healthy monkeys possess genotype I of coagulase gene (pattern 600/600). *St. aureus* with coagulase gene of genotype III and VII were most frequently isolated from the lungs of dead monkeys with diagnosed pneumonia. Some researchers supposed that *St. aureus* with prevailing coagulase genotype are more resistant to neutrophils as compared to the ones with rare genotypes [23].

Agr-typing of *St. aureus* demonstrated that *agr* of group IV was detected in more than half of the isolates, while *agr* of groups II and III were detected rarely (Table 4).

The table demonstrates that *agrIII* was detected in two *St. aureus* isolates recovered from the liver and in one isolate recovered from the spleen. In none of the 66 *St. aureus*

Table 3
Types of coagulase gene of *St. aureus* isolated from biomaterials of monkeys

Type of *coa*-gene	Test material (amount)								
	lung ($n = 66$)	liver ($n = 11$)	kidney ($n = 6$)	spleen ($n = 12$)	lymph node ($n = 9$)	caecum ($n = 4$)	pus ($n = 4$)	feces ($n = 18$)	nasal mucosa ($n = 11$)
I	8	1	1	0	0	1	1	9	5
II	11	1	0	0	0	0	1	1	0
III	12	1	0	1	0	2	0	0	1
IV	8	1	1	3	2	0	0	3	0
V	8	1	0	1	2	0	0	3	3
VI	0	1	1	1	0	0	0	0	0
VII	19	5	3	6	5	1	2	2	2

Table 4
Regulatory gene groups identified in *St. aureus* isolated from different biomaterials of monkeys

Test material	*agr*-type, quantity (%)				Total
	I	II	III	IV	
Lung	22 (33.3%)	3 (4.6%)	0	41 (62.1%)	66
Liver	4 (36.4%)	0	2 (18.2%)	5 (45.4%)	11
Kidney	2 (33.3%)	0	0	4 (66.7%)	6
Spleen	4 (33.3%)	0	1 (8.3%)	7 (58.4%)	12
Lymph node	4 (44.5%)	0	0	5 (55.6%)	9
Uterus	0	0	0	1	1
Pus	1	2	0	1	4
Body fluid	0	0	0	1	1
Caecum	2	1	0	1	4
Feces	5 (27.7%)	1 (5.6%)	0	12 (66.7%)	18
Nasal mucosa	4	1	0	6	11
Throat	0	0	0	1	1
Urethra	0	0	0	1	1
Total	48 (33.1%)	8 (5.5%)	3 (2.1%)	86 (59.3%)	145

isolated from the lungs of the monkeys the regulatory gene of group III was detected, and the isolates recovered from the liver and spleen did not demonstrate *agrII*.

The frequency of detection of *agr* complex genes in *St. aureus* isolates with different types of coagulase gene is shown in Table 2. Analysis of *agr*-typing results demonstrated that in all isolates with Type VII *coa*-gene only group I regulatory gene was detected. Group IV *agr* prevailed in all remaining staphylococci.

CONCLUSION

Use of coagulase gene typing, therefore, allowed for the detection of different genotypes among *St. aureus* isolated from various monkey species. This fact confirms that *St. aureus* has certain heterogenicity in 3´-terminal region of *coa*-gene. Four types of 600–900 bp coagulase gene and seven its restriction profiles with the fragments varying from 80 to 550 bp in length were detected during the studies. Type IV coagulase gene of 900 bp in length was the most wide spread and it was detected in 46% of the isolates, as well as genotype VII *coa*-gene restriction (pattern 900/550-220-80 bp) detected in 31.7% of *St. aureus*. The obtained results demonstrate that pneumonia in monkeys are induced by *St. aureus* strains exhibiting wide coagulase gene polymorphism; however, the majority of *St. aureus* isolates recovered from the lungs (19 of 66) were classified in coagulase gene restriction profile VII (28.8%). There search results suggest that at least seven *St. aureus* isolates circulate in monkeys kept in the monkey colony and these microbes are able to induce pneumonia; they can be part of the concomitant microflora during intestinal diseases (thus persisting both in the intestines and internal organs) and colonize nasal cavity of the clinically healthy animals.

The *agr*-typing demonstrated that group IV regulatory gene prevailed in *St. aureus* isolated from monkeys, while *agrI* prevailed in *St. aureus* isolated from humans, and *agrI* and *agrIII* prevailed in *St. aureus* recovered from the mastitis-diseased cows [4, 6, 25]. Spread of other *St. aureus* virulence determinants is to be further studied, and the above mentioned genes are to be sequenced that will allow to identify whether they belong to the clonal lineages of *St. aureus* circulating in monkeys, to compare them with the data resulted from the examination of the strains isolated from humans and other animals and to assess the epidemic situation in the monkey facilities in terms of this bacterial pathogen.

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Prospects for the use of a *Bacillus subtilis* metabolites-based feed additive in dairy farming

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SUMMARY
Laboratory and field experiment on use of the feed additive based on *Bacillus subtilis* endo- and exometabolites for the cows in different physiological periods is described in the paper. The feed additive impact on main body systems of the tested cows ($n = 30$), milk production parameters, growth rate of the calves ($n = 18$) born to the said cows were examined. The feed additive was added to the diets for the cows of test groups, 15 g per cow. The feed additive was found to have a positive effect on immunohematological and metabolic processes in postpartum cows. Neutrophils' phagocytic activity increased by 12.5 and 14.6% in the animals of test group 1 and test group 2, respectively, as compared to that one in control animals (42.8 ± 1.9%). Neutrophil absorbency increased by 2.5 times, 3.2 times and 2.1 times in the animals of test group 1, test group 2 and control group, respectively. The proportion of T-lymphocytes in blood of animals in test group 1 and test group 2 was 44.5 and 48.9%, respectively, proportion of T-lymphocytes in blood of control animals equaled to 37.5%. Trend for increase in total protein concentration in cow sera owing to increase in albumin fraction was observed in postpartum period: it was 72.91 ± 3.45 g/L in test group 1; 75.54 ± 4.12 g/L in test group 2; 70.95 ± 4.25 g/L in control group. Average daily milk yield in cows of test group 1, test group 2 and control group for 150 days of lactation was 24.50 ± 1.86 kg; 25.33 ± 1.45 kg and 22.75 ± 4.41 kg, respectively. Higher growth rate was reported for the calves born to the cows received the diet supplemented with the said feed additive. Heifers of test group 1 and test group 2 have reached body weight of 193.51 ± 5.76 and 195.33 ± 3.76 kg and in control group – of 187.33 ± 4.98 kg within 6 months. Feed additive based on endo- and exometabolites of *Bacillus subtilis* is recommended for cow diets for high-yielding dairy herd creation and food-producing animal health maintenance.

Keywords: dairy farming, feed additive, *Bacillus subtilis*, immunohematology, biochemistry daily body weight gain

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Перспективы применения кормовой добавки на основе метаболитов *Bacillus subtilis* в молочном животноводстве

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РЕЗЮМЕ

Представлены результаты научно-производственного опыта применения кормовой добавки на основе эндо- и экзометаболитов *Bacillus subtilis* коровам в разные физиологические периоды. Изучено ее влияние на функционирование основных систем организма подопытных животных ($n = 30$), показатели молочной продуктивности, интенсивность роста родившихся от них телят ($n = 18$). Коровам из опытных групп вводили в рацион кормовую добавку в дозе 15 г в сутки на 1 голову. Установлено, что кормовая добавка оказывает положительное влияние на нормализацию иммуногематологических и метаболических процессов у коров в послеродовой период. Показатель фагоцитарной активности нейтрофилов у животных 1-й опытной группы увеличился на 12,5%, 2-й опытной группы – на 14,6% по сравнению с контрольной группой (42,8 ± 1,9%). Поглотительная способность нейтрофилов у особей 1-й опытной группы увеличилась в 2,5 раза, 2-й опытной группы – в 3,2 раза, контрольной группы – в 2,1 раза. Содержание относительного количества Т-лимфоцитов в крови животных 1-й и 2-й опытных групп регистрировали на уровне 44,5 и 48,9% соответственно, у особей контрольной группы данный показатель был равен 37,5%. В послеродовой период отмечали тенденцию к увеличению концентрации общего белка в сыворотке крови коров за счет повышения альбуминовой фракции: в 1-й группе – 72,91 ± 3,45 г/л; во 2-й группе – 75,54 ± 4,12 г/л; в контрольной группе – 70,95 ± 4,25 г/л. Установлено, что за 150 дней лактации среднесуточный удой у коров 1-й группы составил 24,50 ± 1,86 кг; 2-й группы – 25,33 ± 1,45 кг; контрольной группы – 22,75 ± 4,41 кг. Зарегистрирована более высокая интенсивность роста телят, рожденных от коров, которым в основной рацион вводили кормовую добавку. Телочки 1-й и 2-й опытных групп за 6 мес. достигли живой массы тела, равной 193,51 ± 5,76 и 195,33 ± 3,76 кг, а особи контрольной – 187,33 ± 4,98 кг. В целях реализации задачи по созданию высокопродуктивного молочного стада и сохранения биологического благополучия сельскохозяйственных животных-продуцентов кормовая добавка на основе комплекса эндо- и экзометаболитов *Bacillus subtilis* рекомендована к применению в рационах кормления коров.

Ключевые слова: молочное животноводство, кормовая добавка, *Bacillus subtilis*, иммуногематология, биохимия, среднесуточный прирост живой массы тела

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INTRODUCTION

Dairy livestock farming is currently one of the fast-paced agricultural subsectors in the Russian Federation. The said subsector development, improvement and economic efficiency as for satisfying dairy product needs of the country's population should be largely fostered by the state support mechanisms optimization, investment attracting, technological efficiency and herd performance improvement [1, 2]. The main way of milk production ramp-up is sustainable management of the animals with world-class genetic potential and raising of high-yielding dairy cows, development and usage of physiologically adequate and economically sound feed preparation and feeding schemes [3, 4].

Increase in domestic milk production driven by animal performance improvement and creating enabling environment for their genetic potential realization is the task that is getting more urgent every day due to dairy cattle population reduction in our country. At the same time, determination of milk and dairy products safety indicators remains urgent for maintaining the national human population health.

Systematic dairy farming development is intimately associated with the designing a system for animal performance improvement as well as for animal health protection. All kinds of methods including application of different products that are not always environmentally-friendly are used for this purpose. Therefore, biotechnological methods and tools for animal farming industry modernization are being increasingly put into practice, especially those associated with use of new feed additives developed on the basis of prebiotics and metabiotics [5, 6]. Metabiotic feed additives contain metabolites or structural components of probiotic microorganisms. Metabiotics stimulate selectively the growth and biological activity of normal gut microbiota. They are targeted to gut functioning normalization through stabilizing effect on the microbiota and eubiosis support, they optimize metabolism and immunity processes that ultimately should result in an increase in animal performance and product quality [6–8].

Table 1
Design of laboratory and production experiment in cows

Group of animals	Experiment design
Test group 1 ($n = 10$)	Basic diet + 15 g of the feed additive administered to each cow for 14 days before calving
Test group 2 ($n = 10$)	Basic diet + 15 g of the feed additive administered to each cow for 14 days before calving and for 14 days after calving
Control group ($n = 10$)	Basic diet

Development and introduction of new domestic feed additives based on bacterial cell metabolites capable of maintaining controlled gut microbiocenosis and not interfering with gut microbiota that are easily producible and stable during their storage is the main task for researchers and developers in biotechnological industry [6, 7, 9].

The study was aimed at testing of feed additive based on *Bacillus subtilis* endo- and exometabolites for its effect on cow main body systems' functioning and cow milk yields as well as on their offspring weight gain rate.

MATERIALS AND METHODS

The study was performed in the Laboratory for Immunology and Pathobiochemistry of the Ural Research Veterinary Institute of the FSBSI UrFASRC, UrB of RAS and in one agricultural holding located in the Ural Federal District in spring-summer period and in summer-autumn period in 2019.

Second- and third-lactation Russian black pied cows with at least 75% Holstein genetics ($n = 30$) and heifers born to them ($n = 18$) were used for the study.

The tested feed additive contained a complex of *Bacillus subtilis* endo- and exometabolites (proteins, amino acids, enzymes, antibiotic substances, structural components of destroyed bacterial cells, etc.).

The following analogues groups of animals were formed for laboratory and field experiment: two test and one control group, 10 cows in the same physiological state – in late dry period per group. All animals were apparently healthy, kept in one holding facility and fed on the diet used in the said holding. Cows of both test groups were fed individually with tested feed additive in addition to the basic diet. The period when the cows were fed with the feed additive (14 days before and after calving) was determined based on the period of maximum metabolic load on the animals' body [10]. Design of laboratory and field experiment is given in Table 1.

The cows were daily observed for their physiological state for 150 days. Control milkings were performed monthly starting from day 15 after calving to assess the milk yields. Milk fat mass fraction was determined with 'CombiFoss FT+' automatic analyzer (Foss, Denmark).

Blood samples for laboratory tests were collected thrice to vacuum tubes; on day 1 after calving (background), 14 and 28 days after calving in morning hours before feeding.

The experiments in animals were carried out in accordance with the Declaration of Helsinki (2000) and Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on protection of animals used for scientific purposes.

Complete blood count was performed with 'Abacus Junior Vet' hematology analyzer (Diatron, Austria) using standard reagents (Diatron, Austria); white cell count was measured in Romanowsky-Giemsa-stained blood smears (300 cells per smear) under 'Olympus BX 43' microscope (Olympus, Japan). Immunological tests of blood comprised tests for relative T- and B-lymphocyte content, T/B index, phagocyte index (PI), phagocytic activity (PA) of neutrophils and monocytes according to P. N. Smirnov et al. (2007) [11]. The reaction results were viewed under MC 100 (XP) binocular microscope (Micros, Austria) and recorded. Biochemical tests including turbidimetry, colorimetry and kinematic analysis were carried out with 'Chem Well-2910 Combi' analyzer (Awaveness Technology, USA) using original reagent panels (Vital Diagnostics SPb, Russia; DIALAB GmbH, Austria). The reliability of the performed measurements was confirmed by tests of reference materials recommended by the reagent manufacturers.

Three groups of heifers were formed for tests for body weight gains in the offspring of the cow dams involved in the laboratory and field experiment. The design of the test of calves for their body weight gains is given in Table 2.

Arithmetic mean and standard deviation were calculated for all values and expressed as $\bar{x} \pm s.d.$ Calculations and graph plotting were performed with PAST (version 4.05) and MS Excel 2016 packages.

Table 2
Design of the test of heifers for body weight gains

Group of animals	Animal characteristics	Feeding			Frequency of individual weighing
		day 1–5	day 6–15	day 16 and onwards	
Test group 1 ($n = 6$)	Heifers born to the cows of test group 1	Heifers were individually fed thrice with their dams' colostrum	Heifers were individually fed with milk from their dams	Pooled whole milk and basic diet for young animals	At birth; at the age of one month; at the age of two months; at the age of three months; at the age of four months; at the age of five months; at the age of six months
Test group 2 ($n = 6$)	Heifers born to the cows of test group 2				
Control group ($n = 6$)	Heifers born to the cows of control group				

RESULTS AND DISCUSSION

The background tests of the cows of test groups and control group for main morphological blood gradients demonstrated their compliance with standard values for the postpartum period without significant intergroup differences. Tests of blood samples collected from the cows 14 and 28 days after calving showed that erythrocyte, hemoglobin, leukocyte, lymphocyte, platelet counts were variable but were within the normal limits. Dynamics of hematological indicators correlated with hematopoiesis normalization in cows in postpartum period. The experiment findings correlated with data of other researchers [12, 13].

Analysis of the cellular component of the immune system showed that monocyte-macrophage phagocytic activity had increased by day 28 of the experiment as evidenced by increased absorption capacity of phagocytic cells. Neutrophils' phagocytic activity increased by 12.5% (test group, PA 55.32 ± 1.31%) and 14.6% (test group 2, PA 57.44 ± 1.72%) in tests animals, as compared to that one in control animals (PA 42.80 ± 1.91%). Enhanced neutrophil absorbency was reported in animals of all groups but its dynamics was different. In test group 1, phagocytic index increased by 2.5 times as compared to background one and averaged to 9.61 ± 0.54 c. u. In test group 2, phagocytic index increased by 3.2 times (11.82 ± 1.14 c. u.), and in animals of control group phagocytic index increased by 2.1 times (8.21 ± 0.76 c. u.). Relative T-lymphocyte content in blood of animals of test group 1 and test group 2 were 44.5 and 48.9%, respectively, that was correlated to standard physiological parameters (40–60%). Relative T-lymphocyte content in control animals were 37.5%.

T/B-lymphocyte index in animals of test group 1 and test group 2 was 1.51 ± 0.12 and 2.22 ± 0.09 c. u., respectively, that was indicative of the balance between cellular and humoral immunity components. In control animals humoral immunity component slightly prevailed by day 28 of the experiment, T/B-lymphocyte index was 1.39 ± 0.07 c. u.

Thus, it could be supposed that the feed additive administration had an indirect positive impact on hematopoiesis normalization and natural resistance stabilization in postpartum cows.

Analysis of changes in biochemical parameters in cows of all group after calving revealed a trend for increase in total protein concentration in cow sera owing to increase in albumin fraction. Total protein concentration was 72.91 ± 3.45 g/L in test group 1; 75.54 ± 4.12 g/L in test group 2; 70.95 ± 4.25 g/L in control group by day 28 of the experiment (Figure 1).

Slight fluctuations in cholesterol and triglyceride levels within the reference limits were observed in cows of all groups when their lipid metabolisms were assessed. Thus, in cows cholesterol level was not more than 3.2 mmol/L, triglyceride levels were in the range of 0.2–0.3 mmol/L.

It was also important that alkaline phosphatase enzymatic activity values were positive in cows of test groups. Alkaline phosphatase level was by 20.56% and 29.46% lower in cows of test group 1 and test group 2 as compared to that one in cows of control group on day 28 of the experiment. Aspartate aminotransferase (AsAT) activity in cows of all groups was within the reference limits. Detected changes were indicative of total decrease in toxic load on the cows' hepatobiliary system in the postpartum period.

Mineral metabolism intensity was assessed based on dynamics of calcium and phosphorous levels in cow sera. Thus, cumulative calcium and phosphorous values in sera of test group 1 and test group 2 cows were averagely by 5.66 and 6.67% higher than that ones in cows of control group (Figure 1).

It could be supposed that some variability in blood immunobiochemical parameters in cows of test groups were associated with indirect positive impact of the feed additive that was correlated with some research results [2, 12].

Moreover, the additive was noted to have positive effect on accumulation of the significant energy required for animal health and performance maintaining. This was

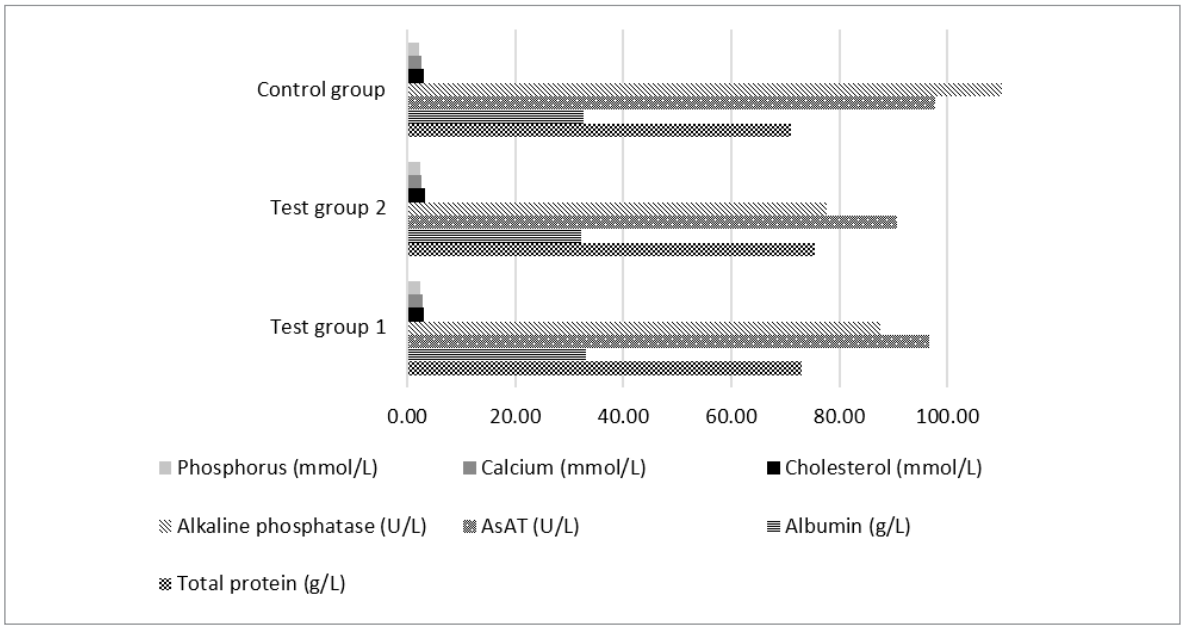

Fig. 1. Biochemical parameters on day 28 of experiment

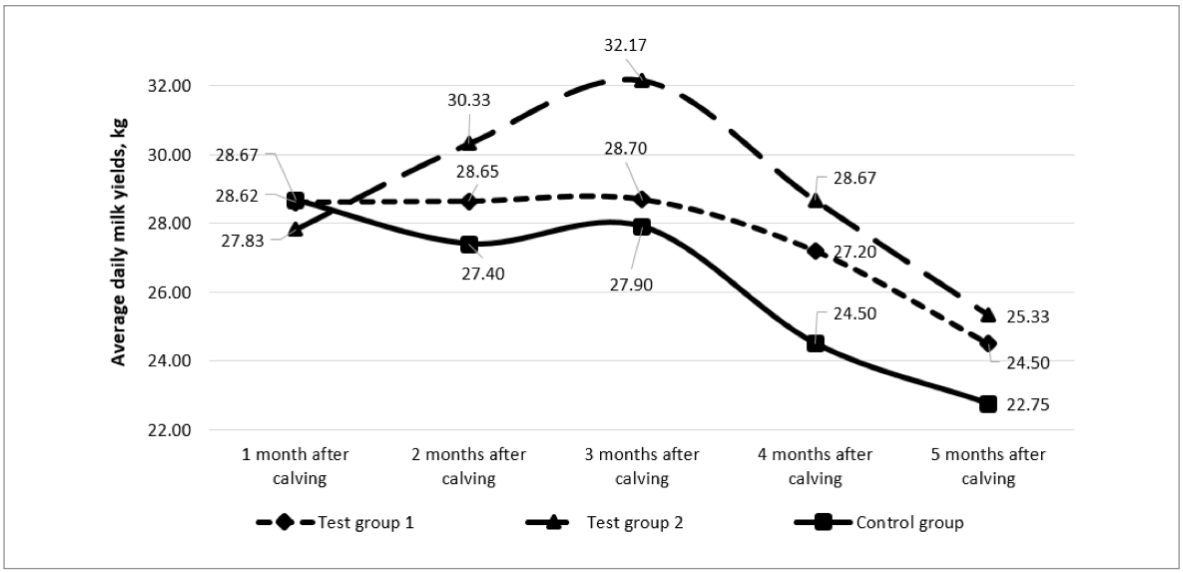

Fig. 2. Dynamics of average daily milk yields (kg) during the experiment

supported by the data on cows' milk yields for the whole test period.

Figure 2 shows graphs of milk yields in all groups. The highest milk yields were registered by day 90 of lactation. Average daily milk yields in cows of test group 1, test group 2 and control group were 28.70 ± 2.65 kg; 32.17 ± 1.92 kg and 27.90 ± 3.24 kg, respectively.

The following average daily milk yields were registered in test group 1, test group 2 and control group by the end of the experiment: 24.50 ± 1.86 kg; 25.33 ± 1.45 kg; 22.75 ± 4.41 kg, respectively.

Milk production levels in the cows fed the diet supplemented with the feed additive remained higher than that ones in the cows of control group during the whole test period starting with the second month after calving.

Mass fraction of protein is an important criterion for milk quality evaluation. The mass fraction of protein varied within the range of 2.85–3.38% during the whole experiment period. The average mass fraction of protein in control group, test group 1, test group 2 was 3.01, 3.03, and 3.27%, respectively, proved the feed additive positive effect on milk quality.

In the view of modern technologies introduced in dairy farming, replacement heifer raising under controlled conditions is of great importance for high-yielding dairy herd creation. It could be achieved through increase in young animals' body weight gain rates that are directly associated with future milk yields [14].

Therewith, average daily body weight gains of future replacement heifers born to the cows fed the feed additive were determined (Figure 3).

The calves born to the cows fed with the feed additive in dry and postpartum periods developed more intensively in the first months of their lives than others under similar

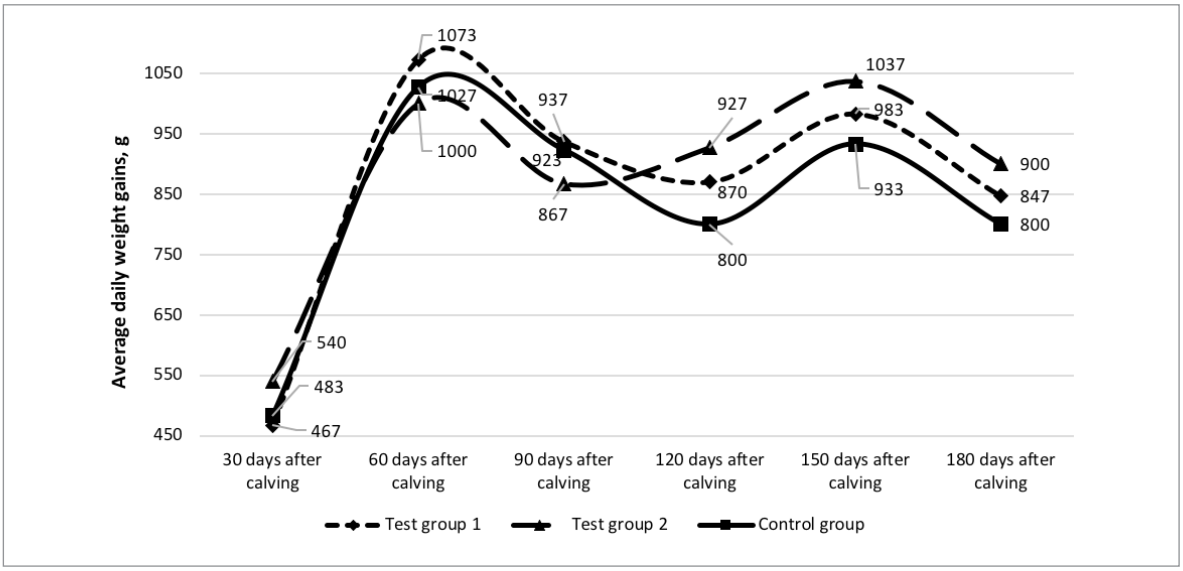

Fig. 3. Dynamics of average daily body weight gains (g) in heifers

feeding and keeping conditions. Average body weight of newborn calves born to the test and control cows was 37.91 ± 1.24 kg. Then, high body weight gain rate was reported in heifers born to the dams fed with a diet supplemented with the feed additive. Thus, the heifers born to the cows of test group 1 and test group 2 had reached body weight of 193.51 ± 5.76 and 195.33 ± 3.76 kg, respectively, for 6 months and the heifers born to control cows had reached body weight of 187.33 ± 4.98 kg for 6 months.

Weight gains in heifers of test groups were higher than that ones in control group during the whole experiment period that confirmed the positive feed additive impact on young animals' growth rate.

The first 6 months of life are proved to be the most important for the internal organ development and functional adjustment, for the development of the ability to absorb nutrients from various feed components, that have indirect impact on the future animal's milk production and growth rate [15, 16]. The experiment data can be accounted for the feed additive positive impact including dams' metabolism normalization, easier calving, more viable less disease-susceptible offspring with more efficient diet nutrient intake. The results of the study are correlated with the data of some authors [17–19].

CONCLUSION

Promising use of *Bacillus subtilis* metabolite complex-based feed additive in dairy farming was demonstrated in the laboratory and field experiment. The said feed additive was shown to have positive impact on cows' hematopoietic and metabolic processes. Thus, blood immunohematological and biochemical parameters had been restored and normalized by day 25–28 regardless of the scheme of the tested feed additive administration to experimental animals in the postpartum period. Protein, lipid and mineral metabolism indicators in the cows of test groups were at the upper normal limit. Analysis of metabolic indicators of hepatobilliary system state showed that the toxic load on parenchymal organs of test animals was minimal on day 28.

Tested *Bacillus subtilis* metabolite complex-based feed additive has a prolonged corrective effect on the cows' intestinal eubiosis manifested by effective diet nutrient absorbance and digestibility. This was confirmed by average daily milk yields in cows of test group 1 and test group 2 that were 24.50 ± 1.86 and 25.33 ± 1.45 kg as compared to that ones in cows of control group that were 22.75 ± 4.41 kg.

Feeding of the heifers born to the feed additive-fed dams with their colostrum and milk had a positive impact on the heifers growth rates (average daily body gains in the heifers born to cows of test group 1 and test group 2 were 862 g and 878 g, respectively; average body weight of the test group 1 and test group 2 at the age of 6 months was 193.51 ± 5.76 kg and 195.33 ± 3.76 kg, respectively).

For creation of a high-yielding dairy herd and maintaining of food-producing animal health it is recommended to use *Bacillus subtilis* endo- and exometabolites-based feed additive in the diets for the cows: 1) in late dry period – at a dose of 15 g/animal for 14 days before calving; 2) in postpartum period for prevention metabolic disorders, general resistance correction and performance improvement – at a dose of 15 g/animal for 14 days after calving.

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Effect of *Chlorella* on hematological parameters and nutrient bioavailability in the diet of rhesus monkeys (*Macaca mulatta*)

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SUMMARY

Chlorella shows a wide spectrum of biological activity, in particular, it exhibits a pronounced antioxidant activity and demonstrates anti-inflammatory, antitumor and antiviral properties. A number of research works have been devoted to studying feed advantages of this unicellular green algae when used in the diets of livestock animals, but the possibility of including different *Chlorella* species in the diet of primates has not been practically studied. The aim of this work was to assess the possibility of replacing high-protein animal and vegetable feeds with *Chlorella*, to calculate the digestibility coefficients for the diet nutrients and the effect of algal dry and suspension forms on hematological and serum biochemical parameters in male rhesus monkeys. The data obtained during the experiment indicate that the inclusion of *Chlorella* in the diet both in the dry form and cell suspension improves nutrient digestibility. Thus, the digestibility of crude protein in the animals receiving algae suspension increased by 4.18% ($p < 0.05$), that of crude fat – by 4.70% ($p < 0.01$), crude fiber – by 4.14% ($p < 0.05$) and crude ash – by 12.32% ($p < 0.001$). The digestibility coefficients of crude protein in the primates receiving compound feed supplemented with *Chlorella* powder were higher by 6.83% ($p < 0.001$), those of crude fiber – by 4.78% ($p < 0.05$) and crude ash – by 18.93% ($p < 0.001$). The hematological study results indicate the absence of side effects from long-term *Chlorella* consumption by primates. The introduction of dry *Chlorella* into the diet increased blood glucose levels to the upper limit of the control values, while *Chlorella* suspension did not produce this effect. Thus, *Chlorella* can be successfully used as a component of a balanced laboratory diet for primates or as a feed additive.

Keywords: *Chlorella*, digestibility, blood composition, biochemical blood test, suspension, primates

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Влияние хлореллы на гематологические показатели и биодоступность питательных веществ рациона у макаков-резусов

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РЕЗЮМЕ

Хлорелла обладает широким спектром биологической активности, в частности, проявляет выраженную антиоксидантную активность, противовоспалительные, противоопухолевые и противовирусные свойства. Изучению кормовых достоинств этой одноклеточной зеленой водоросли при использовании в составе рационов для сельскохозяйственных животных посвящен ряд исследований, однако вопрос возможности включения разных видов *Chlorella* в рацион приматов практически не изучен. Целью данной работы была оценка возможности замещения высокопротеиновых кормов животного и растительного происхождения на хлореллу, определение коэффициентов переваримости питательных веществ рационов и влияния сухой и суспензионной форм водоросли на гематологические, биохимические показатели крови у самцов макаков-резусов. Полученные при проведении эксперимента данные свидетельствуют о том, что включение в рацион хлореллы как в сухом виде, так и в виде клеточной суспензии способствует лучшей усвояемости питательных веществ. Так, в группе животных, получавших суспензию водоросли, усвояемость сырого протеина увеличилась на 4,18% ($p < 0,05$), сырого жира – на 4,70% ($p < 0,01$), сырой клетчатки – на 4,14% ($p < 0,05$) и сырой золы – на 12,32% ($p < 0,001$). У приматов, в рецептуру комбикорма которых был введен порошок хлореллы, коэффициенты переваримости сырого протеина были выше на 6,83% ($p < 0,001$), сырой клетчатки – на 4,78% ($p < 0,05$) и сырой золы – на 18,93% ($p < 0,001$). Результаты гематологических исследований указывают на отсутствие побочных эффектов от длительного употребления хлореллы приматами. Введение в рацион сухой хлореллы способствовало повышению уровня глюкозы в крови до верхней границы контрольных значений, тогда как суспензия хлореллы не оказывала такого эффекта. Таким образом, хлорелла может быть успешно использована в качестве компонента сбалансированного лабораторного рациона для приматов или в качестве кормовой добавки.

Ключевые слова: хлорелла, переваримость, состав крови, биохимический анализ крови, суспензия, приматы

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INTRODUCTION

Of all freshwater algae, the unicellular green algae *Chlorella* (*Chlorella* spp.) is the most widely used and serves a suitable model for laboratory testing and application in production [1]. The diameter of mononuclear vegetative cells of this alga does not usually exceed 15 microns, the protoplast has one cup-shaped chloroplast with a pyrenoid in the thickened part. *Chlorella* species reproduce exclusively by autospores (generally 4–8 per cell) [2]. *Chlorella* has long been used as a food source, it surpasses all plant feeds and agricultural crops in vitamin content, it contains amino acids, including essential ones, which indicates its prospects as a source of valuable organic matter [3]. In addition, microalgae are cultivated in bioreactors using mineral nutrient media, and the grown suspended plant cell culture is used as a genetically modified platform for the production of heterologous proteins [4, 5].

Chlorella actively produces proteins, carbohydrates, lipids, vitamins with an easily adjustable ratio of these compounds when its cultivation conditions are changed. On average, dried algae contain 45% protein, 20% carbohydrates, 20% fat, 5–10% fiber, zinc, iron, magnesium, calcium, phosphorus, etc. Many of the substances found in *Chlorella* accumulate in the culture medium containing vitamin B1 (thiamine), B2 (riboflavin), B3 (pantothenic acid), B5 (nicotinic acid), B6 (pyridoxine), B12 (cyanocobalamin), folic acid and its derivatives, *para*-aminobenzoic acid, H (biotin), inositol [6, 7]. The amount of these vitamins in the culture fluid significantly exceeds their amount in cells. Therefore, when using C*hlorella* biomass as a feed additive, it is also possible to give animals cell suspension with no loss of vitamins and other biologically active substances in the growth medium.

Chlorella shows a wide range of biological activities, in particular, it exhibits pronounced antioxidant activity and has anti-inflammatory, antitumor and antiviral properties [8]. It was found that the alga protects the INS-1 (832/13) cells in the pancreas from damage by hydrogen peroxide, prevents damage to mitochondrial membranes, restores the adenosine triphosphate levels and reduces the cellular content of reactive oxygen species [9]. The constituents composing the *Chlorella* cell wall have immunostimulating properties, which are manifested in increased activity of NK cells, as well as increased production of interferon-γ, interleukin-12, and interleukin-1β – Th1-associated cytokines [10]. *Chlorella* used in humans with viral hepatitis C helps to reduce virus RNA expression, as well as the level of alanine aminotransferase and aspartate aminotransferase [11]. Dried *Chlorella pyrenoidosa* powder induces a chemopreventive effect on hepatocancerogenesis in rats [12]. Both *Chlorella* extracts [13] and polypeptides exhibit antitumor properties [14]. Clinical trials have shown that supplements containing *Chlorella vulgaris* can alleviate hyperlipidemia, hyperglycemia, protect against oxidative stress, as well as prevent development of cancer and chronic obstructive pulmonary disease [3]. In addition, taking *Chlorella* during pregnancy may reduce dioxin content and increase immunoglobulin content in breast milk [15].

Introduction of *Chlorella* suspension in the diet of livestock animals minimizes mortality in young animals, promotes better feed absorption, enhances intestine

peristalsis, preventing stagnation and inflammation of gastrointestinal tract mucosa (diverticulitis), reabsorption of toxic substances, as well as distribution of non-saprophytic microbes [7]. The algae anti-inflammatory and antioxidant properties remove symptoms of ulcerative colitis, irritable bowel syndrome and Crohn's disease. *Chlorella* provides essential nutrients [16, 17], increases body's resistance to infectious diseases, which is especially important when animals are kept outdoors in winter, and is a preventive remedy for vitamin deficiency conditions in the autumn-winter period [7].

Chlorella can be added to the diet of humans and animals in the form of suspension, paste or dry biomass [7]. However, the possibility of including different algae species in the diet of primates has not been practically studied. Taking into account the diverse biological effects of *Chlorella* supplements, it is necessary to evaluate not only the effect on the digestibility of substances, but also on the main hematological and biochemical parameters of the organism.

Thus, the aim of the study was to assess the possibility of replacing high-protein feeds with *Chlorella* of animal and plant origin, to determine the digestibility coefficients for nutrients in diets and the effect of algal dry and suspension forms on hematological and biochemical blood parameters in male rhesus monkeys.

MATERIALS AND METHODS

Animals. Fifteen male rhesus monkeys (*Macaca mulatta*) aged from 7 to 15 years (FSBSI "RIMP" monkey breeding facility) that were kept in individual cells were used in the experiments. According to the method of pairs of analogues and taking into account the age, the animals were divided into three groups – a Control Group and two Experimental Groups (5 animals in each), which received different diets [18].

Animal experiments were performed in accordance with the interstate standards for the maintenance and care of laboratory animals GOST 33215-2014 and GOST 33216-2014, adopted by the Interstate Council for Standardization, Metrology and Certification, as well as in accordance with the requirements of the Helsinki Declaration (2000) and Directive 2010/63/EU of the European Parliament and the Council of the European Union of September 22, 2010 on protection of animals used for scientific purposes. The study was approved by the Bioethical Commission of the FSBSI "RIMP".

The composition of the feed used. Complete granulated compound feed manufactured at the production site of the FSBSI "RIMP" was used in the experiment. In contained wheat – 21.40%, soybean cake – 17.42%, sunflower cake – 13.83%, skimmed milk powder – 14.39%, corn – 13.35%, corn gluten – 11.24%, egg powder – 3.30% and sugar – 4.27%. The diet of the Control Group animals was energetically balanced by adding 0.80% sunflower oil. The biochemical composition of feed and *Chlorella* is shown in Table 1.

Chlorella (*Chlorella vulgaris,* IGF No. C-111 strain) was used in the diets of Experimental Group primates in the form of a suspension at 55–60 million/cm^3 cell concentration (Biocenter Geoflora, LLC, Russia) and dry powder (Green, LLC, Russia). *Chlorella* suspension was administered with drinking water at 2.8 ml/kg of body weight per day in addition to complete compound feed. When *Chlorella* powder was introduced into the diet (13.25%), the content of skimmed milk powder decreased by 90%, and that of egg powder – by 9% in the compound feed formula.

The experimental design is presented in Table 2.

The primates in the first (Control) Group were given complete granulated compound feed and tap water, the animals in the second (Experimental) Group received *Chlorella* suspension included in the diet. The animals in the third (Experimental) Group received compound feed modified with *Chlorella* powder. The experiment lasted 35 days.

At the first stage, the animals were handled to get prepared for the cell keeping conditions for 5 days and the diet was replaced to exclude the influence of previous feeding. The feeding schedule for primates in all groups was the same.

At the second stage, the quantity of consumed feed and excreted faeces was recorded for 5 days. Faeces were collected daily at the same time (morning and evening), weighed and ground in a mortar. 50% of the homogenized mass were taken for testing during each sampling. The collected portions were stored in a refrigerator. After the recording period was over, the initial moisture level was determined in the collected faeces samples by drying to constant weight at 60–70 °C.

The non-organic matter in the biochemical composition of the complete compound feed was determined using a wave-dispersive X-ray fluorescence spectrometer SPECTROSCAN MAX-GVM (NPO "SPECTRON", LLC, Russia) in accordance with the "Method of measuring the mass fraction of Mg, Al, Si, Zn, P, S, Cl, K, Ca, Ba, Ti, Cr, Mn, Fe, Ni, Br, Rb, Sr in powder samples of plant materials by X-ray fluorescence method using X-ray devices for spectral analysis SPECTROSCAN MAX (M-049-RM/12)", FR.1.31.2014.17343. The other indicators were determined using the NIRS DS2500F feed spectral analyzer (FOSS, Denmark).

Clinical and biochemical blood tests. Venous blood and the serum thereof were used for testing. Blood samples (2.5–3.0 ml) were taken from the ulnar or femoral vein on an empty stomach before *Chlorella* supplementation and 35 days post the experiment. Whole blood was stabilized with a heparin solution, and hematological analysis was performed using automatic Coulter AcT 5diff CP analyzer (Beckman Coulter, USA). The level of erythrocytes, leukocytes, platelets, hemoglobin concentration, hematocrit, mean erythrocyte volume, erythrocyte anisocytosis were determined. The erythrocyte sedimentation rate (ESR) was evaluated according to Panchenkov's method.

Blood serum was obtained from anticoagulant-free venous blood that was left in a centrifuge glass tube at a temperature of 15–20 °C until a clot was formed. Decanting and centrifugation was performed with a thin glass rod for 10 min at 1,000–1,500 g. Biochemical analysis (the content of total protein, glucose, total bilirubin, calcium, phosphorus) of non-hemolyzed blood sera was carried out within 2–3 hours after submission using commercial test kits (High Technology, Inc., USA) and a semi-automatic biochemistry analyzer (BioChem SA; High Technology, Inc., USA) in accordance with the manufacturer's instructions.

Statistical analysis. The obtained results were processed statistically using the GraphPad Prizm 8.0 Software (USA) and expressed as arithmetic means and standard errors. The statistical significance of the differences was determined using a one-way dispersion analysis of

Table 1
Complete feed and *Chlorella* powder biochemical composition

Parameters (per 1 kg)	Complete compound feed	Complete compound feed with *Chlorella* powder (13.25%)	*Chlorella* powder
Metabolizable energy (primates), MJ	13.36	13.32	21.14
Dry matter, g	818	813	983
Crude protein, g	269	278	500
Digestible protein (primates), g	227	231	–
Lysine, g	88	91	51
Methionine + cystine, g	6.8	9.3	36
Tryptophan, g	3.3	4.3	15.0
Crude fat, g	70	67	259
Crude fiber, g	39	40	10
Nitrogen-free extracts, g	284	280	356
Starch, g	242	241	–
Sugar, g	192	191	–
Calcium, g	16.3	16.4	11.0
Phosphorus, g	8.8	9.6	18.0
Magnesium, g	2.6	2.6	0.7
Potassium, g	5.9	5.8	9.8
Sulfur, g	2.4	2.3	2.5
Ferrum, mg	75	120	528
Copper, mg	14.5	14.7	5.0
Zinc, mg	20.9	19.0	3.0
Manganese, mg	20.1	20.0	2.2
Cobalt, mg	10.5	10.4	0.2
Iodine, mg	0.18	0.42	3.0
Carotene, mg	1.3	1.3	6.5
Vitamin A, IU	800	158	1,700
Vitamin D, IU	15	14	127
Vitamin E, mg	6.1	6.0	8.7
Vitamin B1, mg	5.2	5.2	2.5
Vitamin B2, mg	3.0	2.9	9.6
Vitamin B3, mg	5.4	5.3	2.2
Vitamin B4, mg	735	717	2,175
Vitamin B5, mg	243.4	241.9	1.6
Vitamin B12, μg	14.3	12.0	11.0

variance followed by *a posteriori* corrections for multiple comparisons in accordance with Tukey's and Sidak's method. The accepted level of statistical significance was $p < 0.05$ [20].

RESULTS AND DISCUSSION

Changes in the blood system are among the first signs of changes occurring in the body as a whole, which is of great diagnostic importance in alimentary disorders [21]. The results of the hematological analysis of primate blood are presented in Table 3.

It was found that the number of leukocytes in the Experimental Groups slightly exceeded the upper limit of the reference values at the beginning of the experiment. At the end of the experiment, no significant changes in the level of leukocytes were observed. The number of erythrocytes and platelets was within the reference values in all groups, both at the beginning and at the end of the experiment, with no significant differences between the groups. The hemoglobin level and the mean hemoglobin content per erythrocyte did not differ between the groups both at the beginning and at the end of the experiment.

Table 2
Experimental design

Groups	Number of animals	Feeding conditions
I Control	5	Complete compound feed
II Experimental	5	Complete feed + *Chlorella* suspension 2.8 ml/kg of body weight
III Experimental	5	Complete feed + dry *Chlorella* content (13.25%). 90% of milk powder and 9% of egg powder were replaced with dry *Chlorella*

ESR values were within the limits of the physiological norm at the beginning of the experiment as well as following *Chlorella* administration. The ESR and leukocyte levels established in the blood indicate absence of inflammatory processes in experimental animals that received both standard and experimental diets.

Thus, during long-lasting *Chlorella* introduction into the diet in suspension or dry powder forms as part of granular compound feeds, there was no significant change in the hemogram indicators both between groups and in terms of reference values.

Some serum biochemical parameters were determined to assess the *Chlorella* effect on metabolism (Table 4).

The glucose level was within the physiological norm in all groups at the beginning of the experiment. At the end of the experiment there was a statistically significant increase in the glucose level to the upper values of the physiological norm in Experimental Group III following dry *Chlorella* introduction into the diet. The test for digestibility coefficient determination (Table 5) of nitrogen-free extracts (NFE) showed no statistically significant increase ($p > 0.05$) in the carbohydrate intake in Group II animals (79.90 ± 2.03%) as compared to the Control Group (78.05 ± 1.12%). However, the NFE digestibility coefficient was the highest in Group III (82.20 ± 2.03%) as compared to the Control Group ($p > 0.05$). It is known that *Chlorella* and its components can produce a hypoglycemic effect [23, 24] as well as increase blood glucose levels in animals [25]. Perhaps the effect of *Chlorella* intake depends on its rate in the diet. Thus, more detailed studies of dry *Chlorella* effect on carbohydrate metabolism and assessment of how it is correlated with the dose in the diet are required.

At the beginning of the experiment the level of bilirubin derived from hemoglobin in bile pigment cells was within the reference values. At the end of the experiment the mean bilirubin level in Experimental Group III (10.38 ± 5.28 μM/L) as compared to the Control Group (6.98 ± 1.47 μM/L) was 48% higher but the differences did not reach statistical significance ($p > 0.05$).

Table 3
Hematological parameters in rhesus monkeys

Parameters	Control Group I		Experimental Group II		Experimental Group III		Reference values*
	at the beginning of the experiment	at the end of the experiment	at the beginning of the experiment	at the end of the experiment	at the beginning of the experiment	at the end of the experiment	
Leukocytes, $\times 10^9$/L	9.8 ± 1.6	10.8 ± 2.0	12.6 ± 1.0	13.4 ± 1.8	11.5 ± 1.6	10.2 ± 1.4	3.1–12.1
Erythrocytes, $\times 10^{12}$/L	6.30 ± 0.05	6.43 ± 0.17	6.31 ± 0.18	5.84 ± 0.09	6.14 ± 0.39	6.14 ± 0.19	4.39–7.02
Hemoglobin, g/L	143.0 ± 1.0	145.0 ± 1.0	145.0 ± 6.0	137.0 ± 4.0	135.0 ± 9.0	137.0 ± 4.0	96.0–143.0
Hematocrit, L/L	0.43 ± 0.01	0.43 ± 0.00	0.43 ± 0.02	0.41 ± 0.01	0.41 ± 0.03	0.41 ± 0.01	0.26–0.47
Mean corpuscular volume, $\times 10^{-15}$ L	67.8 ± 0.4	68.2 ± 0.4	68.4 ± 1.1	69.8 ± 1.0	66.0 ± 0.8	67.2 ± 0.8	67.6–77.5
Mean hemoglobin content per erythrocyte, pg	22.8 ± 0.3	22.8 ± 0.3	22.9 ± 0.5	23.5 ± 0.4	22.0 ± 0.4	22.3 ± 0.4	18.7–26.0
Anisocytosis of erythrocytes, %	13.0 ± 0.2	13.2 ± 0.3	13.3 ± 0.4	13.1 ± 0.4	13.4 ± 0.3	13.6 ± 0.3	12.7–15.2
Platelets, $\times 10^9$/L	308.0 ± 18.0	316.0 ± 26.0	418.0 ± 23.0	366.0 ± 60.0	292.0 ± 48.0	273.0 ± 27.0	155.0–619.0
Mean platelet volume, $\times 10^{-15}$ L	9.7 ± 0.2	9.9 ± 0.3	9.0 ± 0.4	9.8 ± 0.3	9.5 ± 0.4	10.0 ± 0.5	8.0–14.8
ESR, mm/h	0.9 ± 0.1	0.9 ± 0.1	1.1 ± 0.1	1.5 ± 0.3	0.8 ± 0.1	1.1 ± 0.4	0.5–5.0

Data are presented as mean and standard error, $n = 5$ for all groups.

* reference values are given based on B.-S. Koo et al. [22].

Table 4
Serum biochemical parameters in primates

Parameters	Control Group I		Experimental Group II		Experimental Group III		Reference values*
	at the beginning of the experiment	at the end of the experiment	at the beginning of the experiment	at the end of the experiment	at the beginning of the experiment	at the end of the experiment	
Glucose, mM/L	4.43 ± 0.44	3.32 ± 0.38	5.46 ± 0.78	3.68 ± 0.33	4.59 ± 0.46	6.73±0.51[a,b]	1.83–6.66
Total bilirubin, μM/L	6.99 ± 2.74	6.98 ± 1.47	8.93 ± 2.77	4.16 ± 0.66	6.87 ± 5.11	10.38 ± 5.28	1.71–11.97
Phosphorus, mM/L	1.17 ± 0.19	0.61 ± 0.12	0.88 ± 0.18	0.44 ± 0.14	1.33 ± 0.17	0.77 ± 0.17	1.06–2.13
Calcium, mM/L	2.56 ± 0.06	2.01 ± 0.17	2.47 ± 0.06	2.04 ± 0.10	2.65 ± 0.11	2.75 ± 0.12	1.75–2.45
Total protein, g/L	89.00 ± 8.00	80.00 ± 2.00	86.00 ± 3.00	82.00 ± 5.00	104.00 ± 3.00[c]	89.00 ± 3.00	39.00–78.00

Data are presented as mean and standard error, $n = 5$ for all groups.
* reference values are given based on B.-S. Koo et al. [22];
[a] $p < 0.001$ as compared to Control Group;
[b] $p < 0.05$ as compared to the beginning of the experiment;
[c] $p < 0.05$ as compared to Experimental Group II.

Calcium and phosphorus levels in the blood of animals in all three groups were within the reference values at the beginning of the experiment. By the end of the experiment these parameters did not change statistically significantly, but the phosphorus level was below the reference limits in all groups (Table 4) despite higher phosphorus digestibility coefficients in Groups II and III (Table 5).

The protein levels in the blood of animals both at the beginning and at the end of the experiment were higher than the reference values in all groups (Table 4). At the beginning of the experiment higher protein levels were observed in sera of Group III (104.00 ± 3.00 g/L) animals as compared to the Control Group (89.00 ± 8.00 g/L, $p > 0.05$). Increased serum protein levels in Group II at the beginning of the experiment were probably transient and were not associated with *Chlorella* action in the diet of animals.

Daily feed intake and excreted feces were recorded and the chemical composition thereof was analyzed allowing to determine the nutrient digestibility coefficients of various diets (Table 5).

This indicator in the primates of the Experimental Groups was statistically significantly higher than that in the Control Group animals (Table 5). Thus, the digestibility coefficients of crude protein were higher by 4.18% ($p < 0.05$), those of crude fat – by 4.70% ($p < 0.01$), crude fiber – by 4.14% ($p < 0.05$), crude ash – by 12.32% ($p < 0.001$) in Group II animals that received *Chlorella* suspension. In Group III where *Chlorella* powder was included in the diet composition the digestibility coefficients of crude protein were higher by 6.83% ($p < 0.001$), crude fiber – by 4.78% ($p < 0.05$), crude ash – by 18.93% ($p < 0.001$).

The obtained results indicate that the nutrient digestibility improved when *Chlorella* was introduced in the diet of primates (as compared to traditional feed); these results are consistent with the published study results available on nutrient digestibility when adding *Chlorella vulgaris* microalgae into the diet of Boer goats [25], as well as comply with the previously obtained data on increasing the digestibility of crude protein, crude fiber and calcium accessibility when the diet is enriched with *Chlorella* powder or suspension [26]. Enhancing the digestibility of crude protein, which is the most valuable part of the feed, is the most important effect of introducing *Chlorella* into the diet [27].

CONCLUSION

Thus, the inclusion of dry *Chlorella* as a substitute for high-protein feeds of plant and animal origin in the primate diet composition had a positive effect on the nutrient

Table 5
Digestibility coefficients

Groups	Crude protein, %	Crude fat, %	Crude fiber, %	Crude ash, %	Crude NFE, %	Calcium, %	Phosphorus, %
I (Control)	65.34 ± 1.04	38.09 ± 0.98	25.88 ± 1.01	41.58 ± 0.88	78.05 ± 1.12	44.37 ± 1.94	59.79 ± 2.01
II (Experimental)	69.52 ± 0.95*	42.79 ± 0.79**	30.02 ± 0.90*	53.90 ± 1.00***	79.90 ± 2.03	53.66 ± 1.58**	60.51 ± 1.61
III (Experimental)	72.17 ± 0.96***	40.70 ± 0.87	30.66 ± 1.12*	60.51 ± 1.21***	82.20 ± 2.03	58.17 ± 1.96***	67.86 ± 3.12

Data obtained by averaging five measurements for each animal and the mean for the group ($n = 5$).
* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ as compared to Control Group. Data are presented as mean and standard error.

digestibility. At the same time, the hematological test results generally indicate the safety of long-term (35 days) *Chlorella* supplementation in primates. The preparation of complete granular compound feeds supplemented with *Chlorella* powder allows balancing the diet in terms of nutrients and biologically active substances. Similar patterns are typical for *Chlorella* suspension supplementation: the experiments conducted in this regard showed best results for digestibility parameters, indicating that the use of this green algae suspension can significantly increase the metabolism level.

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РЕФЕРЕНТНАЯ ЛАБОРАТОРИЯ МЭБ ПО ВЫСОКОПАТОГЕННОМУ
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