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

FEDERAL SERVICE FOR VETERINARY AND PHYTOSANITARY SURVEILLANCE (ROSSELKHOZNADZOR)

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ВЕТЕРИНАРИЯ СЕГОДНЯ

РУССКО-АНГЛИЙСКИЙ ЖУРНАЛ

MARCH | МАРТ №1 [36] 2021

THE YEAR OF SCIENCE AND TECHNOLOGY IN THE RUSSIAN FEDERATION

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AND PHYTOSANITARY SURVEILLANCE
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From Editor-in-Chief of "Veterinary Science Today" Journal

Dear readers and colleagues, this issue is devoted to the Day of Russian Science. According to Presidential Decree, the year 2021 was declared the Year of Science and Technology. Every month of the year will focus on a particular subject and will be associated with high priority research areas. During the year, awareness events will be held involving outstanding scholars; educational platforms will be launched; and those who wish to take part in contests will have a chance to do it. One of the targets of the Year of Science and Technology is to inform Russian people on those achievements and scientists that Russia can be proud of.

This year's opening ceremony was held on February 8, i.e. on the Day of Russian Science established with Presidential Decree in 1999. There is a reason to choose this particular date. It was February 8, 1724 when Peter the First signed the Order on Establishing the St.-Petersburg's Imperial Academy of Sciences and Arts.

Science has always been one of the basic national resources, a key factor in the national economic prosperity and future.

The Russian Science has always been at the cutting edge of the global science progress and gave many distinguished researchers and discoveries to the world. Despite economic challenges, Russia is still in the front line of the global science, making major breakthroughs. Outstanding scientists are now working in the country and the world community has a great interest in their progress.

The Day of Science is a professional holiday, which means recognition and respect, appreciation of scientific potential and achievements.

Outstanding, truly unique people are honored on that day; their routine work goes hand in hand with continuous search, new discoveries and journeys to the unknown. Progress in all spheres of social life depends on the people of science, sometimes unpredictable, though, target-oriented.

The history of the All-Union Foot-and-Mouth Disease Research Institute dates back to August 1958. Nowadays it is a Federal State-Financed Institution "Federal Centre for Animal Health" (FGBI "ARRIAH"), which is a reference center that provides scientific and methodological support to the Rosselkhoznadzor, its territorial administrations and subordinated organizations; takes scientific and technical actions to ensure biosafety and freedom of the country from animal diseases.

In the course of time, the FGBI "ARRIAH" has founded its own scientific traditions and schools and has gained international fame. Now it is one of the largest research centres for infectious animal diseases with a wide scope of activities and high international status. Experts of the Centre have created a modern methodological base, which makes it possible to solve problems related to diagnostics and epizootological monitoring; to make prognoses for potential spread of diseases and to prepare recommendations on animal and bird health issues and to directly participate in treatment and prevention.

Photo: Alexandr Plonskiy

The Federal Centre for Animal Health employs 938 people, including 14 Doctors of Science (Veterinary and Biology) and more than 120 Candidates of Science; 28 young, high-qualified PhD students.

Over the last 5 years, the researchers of the Centre have obtained 40 patents on their inventions. The Centre issues "Veterinary and Life", specialized federal newspaper, scientific journal "Veterinary Science Today", which is a peer-reviewed journal recommended by VAC (the Higher Attestation Commission of the Russian Federation); it also annually publishes "Proceedings of the Federal Centre for Animal Health" and materials of scientific conferences. Since the establishment of the Centre, more than 2,000 research articles have been published.

The Centre has come a long way. It expanded its scope of research, changed its structure and provided training and support to many researchers. The progress achieved over the years is the result of hard and coordinated work done by high-qualified specialists who are successfully working now at developing effective tools and methods to control dangerous animal diseases, thus, ensuring biosafety and freedom of the country from animal diseases. With due respect for its traditions, the Centre is committed to its history and counts on the younger generation.

I would like to express my appreciation and thanks to those who contribute to the advancement of science, i.e. to Doctors and Candidates of Science, PhD students, students, staff members of the research center. We pay tribute to the high level of your dedication and professionalism, to your hard work and continuous search for perfection and truth.

Best regards,
Editor-in-Chief
Doctor of Science (Veterinary Medicine)
Artem Ye. Metlin

Photo: Alexander Plonskiy

Schedule of FGBI "ARRIAH" activities tailored to the Year of Science and Technology in the Russian Federation

EVENT	DATE AND VENUE
Advanced training course: "Transboundary animal diseases" (e-learning)	*February, April 2021* Vladimir, FGBI "ARRIAH"
Advanced training course: "Challenging zoonotic diseases" (e-learning)	*March, May 2021* Vladimir, FGBI "ARRIAH"
A course of lectures by leading scientific specialists of the FGBI "ARRIAH" (e-learning)	*during 2021*
International scientific conference dedicated to **60th anniversary of the Post-Graduate Education Unit of the FGBI "ARRIAH",** "Achievements of young scientists – to veterinary practice"	*February 2022* Vladimir, FGBI "ARRIAH"
Webinar 'FGBI "ARRIAH": history and achievements" for veterinary faculties of educational institutions	*May, September 2021* Vladimir, FGBI "ARRIAH"
International Scientific and Practical Conference for Veterinarians of Poultry Farms of the Russian Federation and CIS countries "Topical issues of diagnosis and prevention of avian infectious diseases in industrial poultry farming"	*2021* Suzdal, Pushkarskaya Sloboda Hotel premises
Development of audiovisual products about current scientific achievements of the FGBI "ARRIAH"	*during 2021*
Publication of articles in the federal branch newspaper "Veterinary and Life" and the scientific journal "Veterinary Science Today"	*during 2021*
Development of the FGBI "ARRIAH" advertising information products "Year of Science and Technology 2021"	*during 2021*
Webinar with the participation of invited experts "World Rabies Day"	*September 28, 2021* Vladimir, FGBI "ARRIAH"

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Using spectrometric analysis for indirect estimation of 146S component concentration while measuring FMDV RNA amount

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SUMMARY
Foot and mouth disease has a negative impact on economy due to the high cost of eradication campaigns and stringent measures imposed on domestic and international trade in animal products. Prevention and control measures include mass vaccination of susceptible animals and control of post-vaccination immunity level. Concentration of 146S particles, which are the main component affecting the vaccine immunogenicity, is determined during commercial scale production of FMD vaccines. The paper assesses feasibility of spectrometric analysis for indirect determination of 146S component concentration while measuring amount of FMDV RNA isolated after serological binding. This method is cheap, easy-to-use and makes it possible to determine indirectly concentration of FMDV 146S particles in inactivated vaccine raw materials within 3–4 hours. Study of cultural FMDV suspensions shows that the linear model $C_{146S} = (3.9 \times N_{RNA\ 146S} + 566{,}783{,}689)/280{,}818{,}944{,}837$ makes it possible to estimate FMDV 146S component concentration in the vaccine raw materials with the help of a spectrometric analysis. The actual results obtained in real-time reverse transcription – polymerase chain reaction (rtRT-PCR) were 97.0–99.9% consistent with the expected results of the spectrometric analysis used to determine cultural FMDV 146S component concentration. When compared to a complement fixation test, the actual results were 94.5–99.5% in line with the expected ones. The actual results for positive control were 99.0–99.6% in line with the expected ones. As expected, no FMDV genome or 146S particles were detected in the negative control sample.

Keywords: Foot and mouth disease virus (FMDV) RNA, 146S component concentration, spectrometric analysis.

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Применение спектрометрического способа опосредованной оценки концентрации 146S компонента при определении количества РНК вируса ящура

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РЕЗЮМЕ
Ящур причиняет серьезный экономический ущерб, который выражается в существенных затратах на ликвидацию болезни, введение строгих ограничений, налагаемых на внутреннюю и международную торговлю продукцией животноводства. Комплекс мер для борьбы и профилактики заболевания

предполагает массовую иммунизацию восприимчивых животных, а также контроль уровня напряженности поствакцинального иммунитета. При промышленном изготовлении противоящурных вакцинных препаратов определяют концентрацию 146S частиц, которые являются основным компонентом, влияющим на иммуногенную активность вакцины. В статье представлены результаты оценки возможности применения спектрометрического способа для опосредованного определения концентрации 146S компонента при определении количества РНК вируса ящура, выделенной после серологического связывания. Данный способ является дешевым, простым в исполнении, позволяет опосредованно определять концентрацию 146S частиц вируса ящура в неинактивированном сырье для вакцины в течение 3–4 ч. При исследовании суспензий культурального вируса ящура доказано, что линейная модель вида $C_{146S} = (3{,}9 \times N_{РНК\ 146S} + 566\,783\,689)/280\,818\,944\,837$ с помощью спектрального исследования позволяет оценивать концентрацию 146S компонента вируса ящура в сырье для вакцины. Совпадение фактических результатов полимеразной цепной реакции с обратной транскрипцией в режиме реального времени и ожидаемых результатов по определению концентрации 146S компонента культурального вируса ящура спектрометрическим способом составило 97,0–99,9%. При сравнении с данными, полученными в реакции связывания комплемента, совпадение фактических и ожидаемых результатов соответствовало значениям 94,5–99,5%. Для положительного контроля совпадение фактических и ожидаемых результатов составило 99,0–99,6%. В отрицательном контрольном образце геном и 146S частицы вируса ящура не обнаружены, что также соответствовало ожиданиям.

Ключевые слова: РНК вируса ящура, концентрация 146S компонента, спектрометрический анализ.

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INTRODUCTION

Foot and mouth disease is a highly infectious viral disease primarily affecting wild and domestic cloven-hooved [1]. FMD virus is characterized by high antigenic variability due to changes in surface protein genes; there are 7 serotypes: A, O, C, Asia-1, SAT-1, SAT-2, SAT-3, including many subtypes [2].

The genome is represented by a single-stranded, positive-sense RNA of 8,500 bp. The virion molecular weight ranges between 8,080,000 and 8,167,500 Da [2, 3]. Following infection of biological systems, FMDV forms four types of particles with different sedimentation coefficients: 146S component (whole virus particles) consisting of one viral RNA molecule and 60 copies of VP_1-VP_2-VP_3-VP_4 polypeptide;75S component lacking RNA and including 60 copies of VP_1-VP_3-VP_0 polypeptide; 12S particles represented by proteins VP_1, VP_2, VP_3; 3.8S subunits consisting of functional protein VP_g [4].

FMD causes huge economic losses associated with the costs of the disease eradication and stringent measures imposed on domestic and international trade in animal products. Complex measures for FMD prevention and control include stamping-out, mass immunization of susceptible animals as well as control of postvaccinal immunity level [1, 5].

Concentration of 146S component, which directly affects the vaccine immunogenicity, is determined during commercial scale production of FMD vaccines [2]. A quantitative complement fixation test (CFT) is traditionally used for this purpose; afterwards the results are assessed pursuant to the methodical instructions [6]. However, the test has a number of disadvantages: it is labour-intensive and time-consuming (up to 3 days); it is impossible to simultaneously test a great number of samples, despite it is required by the production process; a high cost of the procedure. A real-time reverse transcription – polymerase chain reaction (rtRT-PCR) has been used in recent years to determine indirectly concentration of FMDV 146S component in non-inactivated suspension [7]. The represented method is highly sensitive, specific and cost-effective; is rapidly performed, provides rapid results and enables to simultaneously test dozens of samples of non-inactivated virus-containing material. However, rtRT-PCR requires expensive chemicals and equipment.

A spectrometric analysis has been developed to indirectly determine concentration of FMDV 146S component in non-inactivated suspensions while measuring amount of the viral RNA isolated after serological binding of the whole particles; with linear model $C_{146S} = (3{,}9 \times N_{RNA\ 146S} + 566{,}783{,}689)/280{,}818{,}944{,}837$ used. The proposed method is cheap, easy-to-use and makes it possible to determine concentration of FMDV 146S particles in the non-inactivated raw materials for vaccine within 3–4 hours [8].

The purpose of the research is to assess the feasibility of the spectrometric analysis for indirect determination of 146S component concentration while measuring amount of FMDV RNA in suspensions.

MATERIALS AND METHODS

Virus. We used in the research non-inactivated cultural FMDV suspensions of the following vaccine production strains: A/Turkey/2006, A/ARRIAH/2015, O/Primorsky/2012, O/Primorsky/2014, Asia-1/Tajikistan/2011, SAT-2/ Saudi Arabia 7/2000. The virus was cultivated in suspension continuous baby hamster kidney cell line (BHK-21/SUSP/ARRIAH) at a concentration of $(3.0–3.5) \times 10^6$ million cells/cm^3.

Plate immobilization and serological binding of FMDV with strain-specific immunoglobulin G (IgG) were carried out in accordance with the requirements provided earlier [8].

RNA extraction from the whole FMDV particles. RNA elution from the FMDV 146S component bound with strain-specific IgG was carried out in accordance with P. Chomczynski's method [9, 10]. As a result, 30-fold viral RNA extracts were obtained (0.1 cm^3 each). These eluates were either immediately used in further activities or were frozen at the temperature of –20 °C.

Using spectrum analysis to measure purity of FMDV RNA eluate. The spectral absorption capacity of RNA extracts was measured in the UV spectrum at wavelength from 205 to 325 nm. The residues of phospholipids, polysaccharides and guanidine isothiocyanate, phenol, polypeptides and large conglomerates were measured by the optical density (OD) values at wavelength of 205, 235, 270, 280 and 320 nm, respectively [11]. The RNA eluate was considered free from protein and carbolic acid impurities if the R_1 extinction coefficient (OD_{262}/OD_{280}) was within the range of 1.8–2.2 and was tended to 2.0. The exceeded value suggested nucleic acid degradation into oligonucleotides and free nucleotides. FMDV RNA extract was considered free from carbohydrates, if R_2 (OD_{262}/OD_{235}) extinction coefficient ranged between 2.00–2.02. If 1% RNA is substituted with polysaccharide components, the R_2 coefficient decreases by 0.002. If $R_2 > 2.02$, dissociation of nucleic acid is observed and free nucleotides or oligonucleotides in the tested sample eluate are reported. The absence of large suspended particles in the FMDV RNA extract was confirmed, if OD_{320} tended to zero [12].

Estimating the number of FMDV RNA molecules. The number of RNA molecules extracted from the whole FMDV particles ($N_{RNA\ 146S}$) was estimated using the following formula:

$$N_{RNA\ 146S} = 0.98 \times \frac{41.67 \times K \times (OD_{262} - OD_{320} - OD_{260\,C-}) \times N_A}{10^{7.48} \times Mw_{ribonucleoside} \times L},$$

where K – dilution factor of the RNA eluate extracted from the whole FMDV particles;

OD_{262} – optical density of the RNA extract isolated from FMDV 146S component at wavelength of 262 nm;

OD_{320} – optical density of the RNA extract isolated from FMDV 146S component at wavelength of 320 nm;

$OD_{260\,C-}$ – optical density of the negative control at wavelength of 260 nm;

N_A – Avogadro's constant (in the SI system (SI), according to redefinition of the SI base units exactly equals to $6.02214076 \times 10^{23}$ mol^{-1});

$Mw_{ribonucleoside}$ – ribonucleoside molecular-weight average (340.5 Da);

L – length of FMDV RNA (about 8,500 bp, pursuant to the data provided by the National Center for Biotechnology Information);

41.67 – FMDV RNA factor ($F_{FMDV\ RNA}$);

$1/10^{7.48}$ – cumulative coefficient for mass conversion from [µg] to [g] and recalculation of FMDV RNA molecule number from the 30-fold eluate into one-fold extract [8].

Determination of 146S particle concentration using the number of FMDV RNA molecules. Based on the data obtained on the number of viral RNA molecules ($N_{RNA\ 146S}$), concentration of FMDV 146S particles (C_{146S}) was determined with the help of the math expression given below: $C_{146S} = (3.9 \times N_{RNA\ 146S} + 566{,}783{,}689)/280{,}818{,}944{,}837$ [8].

In accordance with the relevant requirements, a qualitative CFT was simultaneously carried out to determined concentration of the whole FMDV particles (in µg/cm^3) [6].

Real-time reverse transcription – polymerase chain reaction (rtRT-PCR). FMDV RNA in non-inactivated suspension used for vaccine production was quantified in rtRT-PCR, in accordance with the relevant requirements. Amplification cycle threshold was determined in the test, thus making it possible to estimate indirectly the amount of the virus nucleic acid; and the data obtained were interpreted to determine concentration of FMDV 146S particles [7].

Control samples. Non-inactivated suspension of Asia-1/Tadjikistan/2011 FMDV strain with 146S component at a concentration of 3.7 µg/cm^3 was used as a positive control cultivated in suspension continuous baby hamster kidney cell line (BHK-21/SUSP/ARRIAH). Suspension of BHK-21/SUSP/ARRIAH cell culture at a concentration of $(3.0–3.5) \times 10^6$ million cells/cm^3 was used as a negative control.

Statistical data processing included calculation of arithmetical means, confidence level for statistical difference between mean values determined with differential Student's – Fischer test; calculation of standard deviations in concentration of RNA molecule numbers and the number of the whole FMDV particles. The data were processed and diagrams were drawn using StatSoft (version 6.0) and Microsoft Excel 2010 software application package.

RESULTS AND DISCUSSION

A spectrometric analysis was checked in this research as a way to determine indirectly concentration of 146S particles while FMDV RNA amount is estimated in the strains indicated in "Materials and Methods". Each test included the use of positive and negative control samples indicated above.

At the first stage, concentration of FMDV 146S component of Asia-1/Tajikistan/2011 strain (positive control) was determined in spectrometric analysis while estimating amount of the viral RNA isolated after serological binding with the help of IgG; and in quantitative CFT and rtRT-PCR variants. Immunobinding of the whole virus particles from non-inactivated virus suspension was carried out as well as extraction of RNA from FMDV 146S component bound by strain-specific antibodies. Summary data on purity of the obtained eluates for experimental and control samples are given in the table below.

The spectrum analysis of the 30-fold FMDV RNA eluate of Asia-1/Tajikistan /2011 strain demonstrates that the mean extinction values at wavelengths of 205–259 nm ($OD_{205-259}$) and 263–325 nm ($OD_{263-325}$) did not exceed $OD_{260-262}$ (0.001–0.948 < 0.951–0.952 and 0.947–0.002 < 0.951–0.952), that means the preparation contained mainly ribonucleic acid. The eluate did not contain phospholipids, polysaccharides and guanidine isothiocyanate residues, carbolic acid, proteins and large conglomerates, as there were no marked peaks in the graphs at wavelengths of 205, 235, 270, 280 and 320 nm, correspondingly. The R_1 extinction coefficient was 1.996 (OD_{262}/OD_{280} = 0.952/0.477) which is close to norm of 2.000 and demonstrates high purity of the eluate as well as almost total absence of polypeptide components and residues of carbolic acid after RNA extraction. The R_2 extinction coefficient was 2.000 (OD_{262}/OD_{235} = = 0.952/0.476) which corresponded to norm and accounted for high purity of the control preparation, thus, increasing reliability of the carried out test [12].

The spectrometric analysis of FMDV RNA demonstrated the following mean optical density values:

OD_{262} = 0.952 ± 0.001, OD_{320} = 0.002, OD_{260C-} = 0.006 (for OD_{320} and OD_{260}). Arithmetic mean of the RNA molecules (number of structural particles) isolated from FMDV 146S component of Asia-1/Tajikistan/2011 strain was 265,600,889,380 ± 331,586,628. Using the developed linear bond model C_{146S} and $N_{RNA\ 146S}$ [8], concentration of the analyte was indirectly determined in the positive control (3.691 ± 0.004 μg/cm³) and it had 99.43% and 99.84% correlation with the results of quantitative CFT (3.710 ± 0.170 μg/cm³) and rtRT-PCR (3.700 ± 0.030 μg/cm³), correspondingly. Thus, the positive control met the requirements for purity and for the declared amount of the 146S component. None of the used methods detected RNA or FMDV 146S particles in the negative control.

Concentration of FMDV 146S component was simultaneously determined in experimental samples of the following strains: A/Turkey/2006, A/ARRIAH/2015, O/Primorsky/2012, O/Primorsky/2014, Asia-1/Tajikistan/2011, SAT-2/Saudi Arabia 7/2000 with the help of a spectrometric analysis while estimating the amount of FMDV RNA eluated after serological binding and with the help of quantitative CFT and rtRT-PCR. The test stages were carried out in accordance with procedures described above. The figure and the table provide results of the purity estimation for the RNA eluates of the abovementioned FMDV strains.

The spectrum analysis of the 30-fold FMDV RNA eluate of A/Turkey/2006 strain demonstrates that mean extinction values at wavelengths of 205–259 and 263–325 nm did not exceed $OD_{260\text{-}262}$ (0.001–0.482 < 0.483–0.488 and 0.487–0.002 < 0.483–0.488), which means the preparation contained mainly RNA molecules. The eluate was not contaminated with phospholipids, polysaccharides or guanidine isothiocyanate residues, phenol, protein components or large conglomerates, as there were no marked peaks in the graphs at wavelengths of 205, 235, 270, 280 and 320 nm, correspondingly. The R_1 extinction coefficient was 1.984 (OD_{262}/OD_{280} = 0.488/0.246) which is close to norm of 2.000 and meant almost total absence of polypeptide components and residues of carbolic acid after RNA extraction. The R_2 extinction coefficient was 1.992 (OD_{262}/OD_{235} = 0.488/0.245) which is close to 2.000 and accounts for high purity of the eluate. When 1% RNA was substituted with polysaccharides, the R_2 coefficient decreased by 0.002, i.e. polysaccharide level in the obtained extract did not exceed 4% and it is admissible (not more than 10%) [12].

The spectrum analysis of the FMDV RNA extract of A/Turkey/2006 strain demonstrated the following mean values of optical density: OD_{262} = 0.488 ± 0.001, OD_{320} = 0.002, OD_{260C-} = 0.006 (for OD_{320} and OD_{260}). Arithmetic mean of the structural particles (RNA) isolated from the whole FMDV particles was 135,051,299,685 ± 281,356,875. Using the developed linear bond model C_{146S} and $N_{RNA\ 146S}$ [8] concentration of the 146S component was calculated and it was 1.878 ± 0.004 μg/cm³ and it had 98.84% and 99.89% correlation with the results of quantitative CFT (1.900 ± 0.180 μg/cm³) and rtRT-PCR (1.895 ± 0.032 μg/cm³), correspondingly. Thus, the previously developed linear model makes it possible (with the help of a spectrum analysis) to determined concentration of FMDV 146S component in the non-inactivated suspension for vaccine production.

The spectrum analysis of the 30-fold FMDV RNA eluate of A/ARRIAH/2015 strain demonstrated that mean extinction values at wavelengths of 205–259 and 263–325 nm did not exceed $OD_{260\text{-}262}$ (0.001–0.403 < 0.404–0.407 and 0.403–0.001 < 0.404–0.407), which means that the obtained preparation contained mainly ribonucleic acid. The extract did not contain phospholipids, polysaccharides or guanidine isothiocyanate residues, phenol, polypeptides or large suspended particles, as there were no marked peaks in the graphs at wavelengths of 205, 235, 270, 280 and 320 nm, correspondingly. The R_1 extinction coefficient was 1.995 (OD_{262}/OD_{235} = 0.407/0.204) which is close to norm of 2.000 and meant almost total absence of protein and carbolic acid impurities. The R_2 extinction coefficient was 1.995 (OD_{262}/OD_{280} = 0.407/0.204) which is close to 2.000 and accounts for high purity of the extract. When 1% RNA was substituted with polysaccharide components, the R_2 coefficient decreased by 0.002, i.e. polysaccharide components in the obtained preparation did not exceed 2.5% and it is admissible [12].

The spectrum analysis of the FMDV RNA eluate of A/ARRIAH/2015 strain demonstrated the following mean values of optical density: OD_{262} = 0.407 ± 0.001, OD_{320} = 0.003, OD_{260C-} = 0.006 (for OD_{320} and OD_{260}). Arithmetic mean of the RNA molecules eluated from FMDV 146S particles after strain-specific serological binding resulting from neutralization test was 111,698,679,114 ± 281,356,870. Concentration of FMDV 146S component in the obtained sample was 1.553 ± 0.004 μg/cm³ and it had 98.29% and 99.94% correlation with the results of quantitative CFT (1.58 ± 0.17 μg/cm³) and rtRT-PCR (1.555 ± 0.041 μg/cm³), correspondingly. Thus, the above-mentioned linear mathematical function makes it possible (with the help of a spectrum analysis) to determined concentration of the whole FMDV particles in the non-inactivated suspension for production of a large range of vaccines [8].

The spectrum analysis of the FMDV RNA eluate of A/Primorsky/2014 strain demonstrated that mean values of $OD_{205\text{-}259}$ and $OD_{263\text{-}325}$ did not exceed $OD_{260\text{-}262}$ (0.001–0.311 < 0.312–0.314 and 0.311–0.001 < 0.312–0.314), which means that the obtained extract contained mainly ribonucleic acid molecules. The eluate was not contaminated with phospholipids, polysaccharides or guanidine isothiocyanate residues, carbolic acid, polypeptides or large aggregated particles, as there were no marked peaks in the graphs at the wavelengths of 205, 235, 270, 280 and 320 nm, respectively. The R_1 extinction coefficient was 1.987 (OD_{262}/OD_{280} = 0.314/0.158) which is close to norm of 2.000 and means almost total absence of peptides and residues of carbolic acid in the extracted RNA. The R_2 extinction coefficient was 2.000 (OD_{262}/OD_{235} = 0.314/0.157) which is close to norm and accounts for the absence of polysacciride impurities and signs of viral RNA degradation [12].

The spectrum analysis of the FMDV RNA eluate of A/Primorsky/2014 strain demonstrated the following mean values of optical density: OD_{262} = 0.314 ± 0.002, OD_{320} = 0.002, OD_{260C-} = 0.006 (for OD_{320} and OD_{260}). The average number of RNA structural particles in the extract was 86,095,203,549 ± 562,713,749, concentration of viral 146S component was 1.198 ± 0.008 μg/cm³ which had 99.01% and 99.50% correlation with the results of quantitative CFT (1.21 ± 0.15 μg/cm³) and rtRT-PCR (1.999 ± 0.045 μg/cm³), correspondingly. Thus, the earlier developed linear mathematical model makes it possible (with the help of a spectrum analysis) to determined concentration of FMDV 146S component in the non-inactivated raw materials for production of FMD vaccines [8].

Table
Correlation between results of indirect 146S concentration determination in the tested samples based on the number of FMDV RNA molecules and correlation with CFT and rtRT-PCR data ($n = 3, p < 0.005$)

Таблица
Соотношение результатов опосредованного определения концентрации 146S частиц исследуемых образцов по количеству молекул РНК вируса ящура и корреляции с данными РСК и ОТ-ПЦР-РВ ($n = 3, p < 0,005$)

FMDV strain	Spectrum analysis of RNA eluate						$OD_{260C\text{-}mean}$	$N_{RNA\ 146Smean}$	$C_{146Smean}$, μg/cm³		
	$OD_{262mean}$	$OD_{235mean}$	$OD_{280mean}$	$OD_{320mean}$	R_1	R_2			Spectrum analysis*	CFT [6]	rtRT-PCR [7]
Asia-1/Tajikistan/2011	0.810 ± 0.001	0.407 ± 0.001	0.406 ± 0.001	0.003 ± 0.000	1.995	2.000	0.006 ± 0.000	225,366,856,349 ± 281,356,874	3.132 ± 0.004	3.150 ± 0.170	3.137 ± 0.040
A/Turkey/2006	0.488 ± 0.001	0.245 ± 0.002	0.246 ± 0.001	0.002 ± 0.000	1.984	1.992	0.006 ± 0.000	135,051,299,685 ± 281,356,871	1.878 ± 0.004	1.900 ± 0.180	1.876 ± 0.040
O/Primorsky/2014	0.314 ± 0.002	0.157 ± 0.001	0.158 ± 0.001	0.002 ± 0.000	1.987	2.000	0.006 ± 0.000	86,095,203,549 ± 562,713,749	1.198 ± 0.008	1.210 ± 0.150	1.204 ± 0.050
A/ARRIAH/2015	0.407 ± 0.001	0.204 ± 0.001	0.204 ± 0.001	0.003 ± 0.000	1.995	1.995	0.006 ± 0.000	111,698,679,114 ± 281,356,870	1.553 ± 0.004	1.580 ± 0.170	1.554 ± 0.041
O/Primorsky/2012	0.339 ± 0.002	0.170 ± 0.001	0.171 ± 0.001	0.001 ± 0.000	1.982	1.994	0.006 ± 0.000	93,410,482,282 ± 562,713,747	1.299 ± 0.008	1.330 ± 0.160	1.314 ± 0.050
SAT-2/Saudi Arabia 7/2000	0.203 ± 0.002	0.102 ± 0.001	0.103 ± 0.001	0.001 ± 0.000	1.971	1.990	0.006 ± 0.000	54,864,590,497 ± 562,713,752	0.764 ± 0.008	0.750 ± 0.140	0.768 ± 0.040
Asia-1/Tajikistan/2011 (positive control)	0.952 ± 0.001	0.476 ± 0.001	0.477 ± 0.001	0.002 ± 0.000	1.996	2.000	0.006 ± 0.000	265,600,889,380 ± 331,586,628	3.691 ± 0.004	3.710 ± 0.170	3.700 ± 0.030

$OD_{262mean}$ – mean extinction for 262 nm; $OD_{235mean}$ – mean extinction for 235 nm; $OD_{280mean}$ – mean extinction for 280 nm;
$OD_{320mean}$ – mean extinction for 320 nm; $OD_{260C\text{-}mean}$ – mean extinction of negative control for 260 nm;
R_1 – extinction coefficient (OD_{262}/OD_{280}), calculated based on OD mean values at the wavelengths of 262 and 280 nm;
R_2 – extinction coefficient (OD_{262}/OD_{235}), calculated based on OD mean values at the wavelengths of 262 and 235 nm;
$N_{RNA\ 146Smean}$ – average number of RNA molecules, isolated from FMDV 146S particles; $C_{146Smean}$ – mean concentration of FMDV 146S particles;
* The proposed spectrometric method to indirectly determine concentration of the whole FMDV particles [8].

$OD_{262ср.}$ – среднее значение экстинкции при 262 нм; $OD_{235ср.}$ – среднее значение экстинкции при 235 нм; $OD_{280ср.}$ – среднее значение экстинкции при 280 нм;
$OD_{320ср.}$ – среднее значение экстинкции при 320 нм; $OD_{260К\text{-}ср.}$ – среднее значение экстинкции отрицательного контроля при 260 нм;
R_1 – коэффициент экстинкции (OD_{262}/OD_{280}), рассчитанный исходя из средних значений OD при длинах волны 262 и 280 нм;
R_2 – коэффициент экстинкции (OD_{262}/OD_{235}), рассчитанный исходя из средних значений OD при длинах волны 262 и 235 нм;
$N_{РНК\ 146Sср.}$ – среднее количество молекул РНК, выделенных из 146S частиц вируса ящура; $C_{146Sср.}$ – среднее значение концентрации 146S частиц вируса ящура;
* Предложенный спектрометрический способ опосредованного определения концентрации полных частиц вируса ящура [8].

The spectrum analysis used for indirect determination of 146S particles concentration when number of FMDV RNA molecules was calculated in A/Primorsky/2012 strain demonstrated that mean values of $OD_{205-259}$ and $OD_{263-325}$ did not exceed $OD_{260-262}$ (0.002–0.335 < 0.336–0.339 and 0.334–0.001 < 0.336–0.339) which means the extract contained mainly RNA molecules. The eluate was not contaminated with phospholipids, carbohydrate components or guanidine isothiocyanate residues, hydroxybenzene, proteins or conglomerates, as there were no marked peaks in the graphs at wavelengths of 205, 235, 270, 280 and 320 nm, correspondingly. The R_1 extinction coefficient was 1.982 (OD_{262}/OD_{280} = 0.339/0.171) which is close to norm of 2.000 and means almost total absence of polypeptides, peptides or residues of carbolic acid in the analyzed extract. The R_2 extinction coefficient was 1.994 (OD_{262}/OD_{235} = 0.339/0.170) which is close to norm of 2.000. The data obtained explain the absence of signs of viral RNA degradation and the amount of polysacciride impurities not exceeding 3% (which is admissible) [12].

Based on the results of the spectrum analysis of the RNA eluate, the following mean values of optical density were obtained: OD_{262} = 0.339 ± 0.002, OD_{320} = 0.001, OD_{260C-} = 0.006 (for OD_{320} and OD_{260}). The average number of RNA molecules in the sample was 93,410,482,282 ± 562,713,747, and concentration of the whole FMDV particles in A/Primorsky/2012 strain was 1.299 ± 0.008 μg/cm³ which had 97.67% and 98.66% correlation with the results of quantitative CFT (1.33 ± 0.16 μg/cm³) and rtRT-PCR (1.302 ± 0.052 μg/cm³), correspondingly. Thus, the mathematical expression used in the research (with the help of a spectrum analysis) makes it possible to determined concentration of FMDV component with 146S sedimentation coefficient in the non-inactivated suspension for FMD vaccines [8].

The spectrum analysis of the 30-fold FMDV RNA eluate of Asia-1/Tajikistan /2011 strain demonstrates that the mean $OD_{205-259}$ and $OD_{263-325}$ values did not exceed $OD_{260-262}$ (0.001–0.801 < 0.802–0.810 and 0.801–0.003 < < 0.802–0.810) that means the eluate contained mainly ribonucleic acid. The extract did not contain phospholipids, carbohydrates or residues of guanidine isothiocyanate detergent, hydroxybenzene, polypeptides or suspended conglomerates, as there were no marked peaks in the graphs at wavelengths of 205, 235, 270, 280 and 320 nm, correspondingly. The R_1 extinction coefficient was 1.995 (OD_{262}/OD_{280} = 0.810/0.406) which is close to norm of 2.000 and means high purity of the RNA eluate and almost total absence of polypeptide components or residues of carbolic acid. The R_2 extinction coefficient was 1.990 (OD_{262}/OD_{235} = 0.810/0.407) which is also close to 2.000 and accounts for high purity of the sample. When 1% RNA was substituted with polysaccharide components, the R_2 coefficient decreased by 0.002, therefore, the obtained extract contained not more than 5% of carbohydrate components, which is admissible [12]. Thus, the RNA eluate isolated from FMDV Asia-1/Tajikistan/2011 strain was highly pure, which made it possible to determine

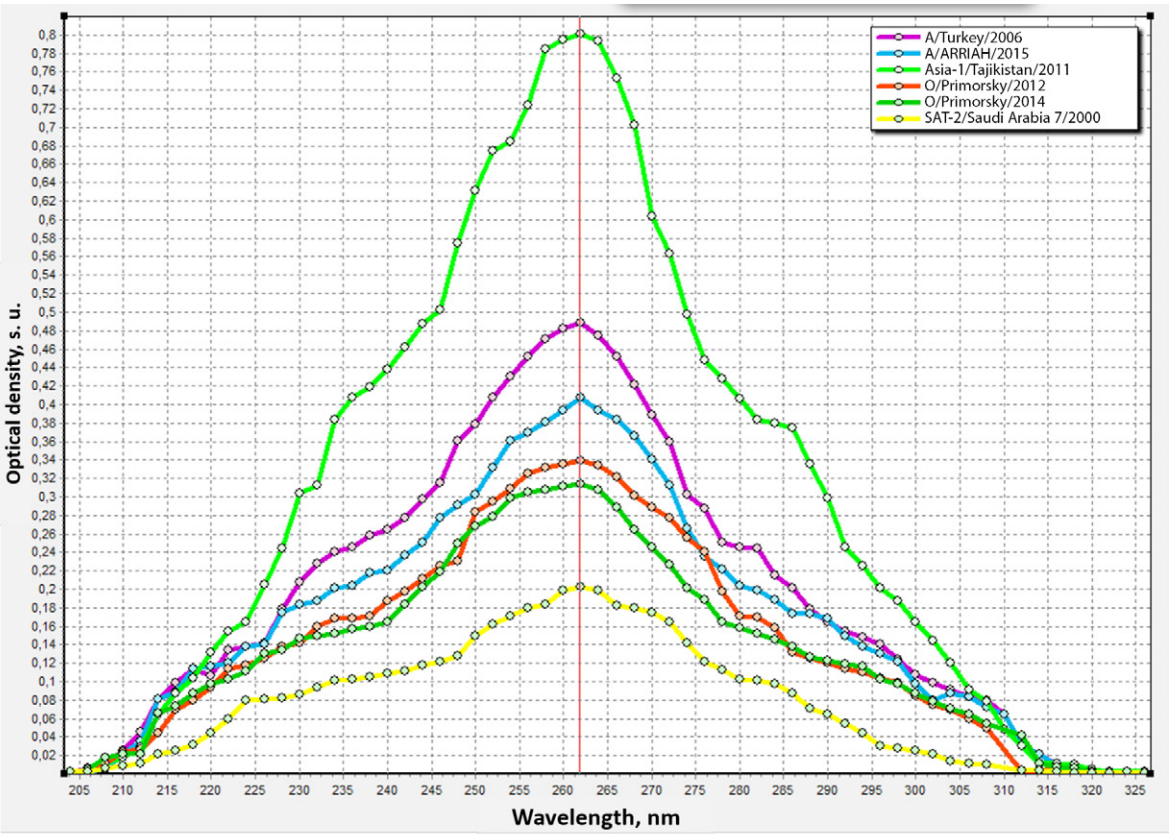

Fig. Spectrograms of the FMDV RNA extracts of the studied strains to assess purity and determine concentration of 146S particles in the original suspensions

Рис. Спектрограммы экстрактов РНК вируса ящура исследуемых штаммов для оценки чистоты и определения концентрации 146S частиц в исходных суспензиях

the number of structural particles (RNA molecules) with a high degree of reliability.

Based on the results of the spectrum analysis, the following mean values of optical density OD_{262} were obtained: 0.810 ± 0.001, OD_{320} = 0.003, OD_{260C-} – 0.006 (for OD_{320} and OD_{260}). Arithmetic mean of the structural particles (RNA) isolated from the whole FMDV particles was 225,366,856,349 ± 281,356,874, concentration of FMDV 146S component of Asia-1/Tajikistan/2011 strain was 3.132 ± 0.004 μg/cm^3 and it had 99.49 and 99.76% correlation with the results of CFT (3.150 ± 0.170 μg/cm^3) and rtRT-PCR (3.136 ± 0.040 μg/cm^3), correspondingly. Thus, the linear algebraic bond function of C_{146S} and $N_{RNA\ 146S}$ developed on the basis of spectrum analysis data makes it possible to determined concentration of the whole particles in the non-inactivated suspension for FMD vaccines [8].

The spectrum analysis of the 30-fold FMDV RNA eluate of SAT-2/Saudi Arabia 7/2000 strain demonstrated that mean extinction values at wavelengths of 205–259 and 263–325 nm did not exceed $OD_{260\text{-}262}$ (0.002–0.199 < < 0.200–0.203 and 0.198–0.001 < 0.200–0.203), which means that the eluate contained mainly ribonucleic acid. The extract was not contaminated with phospholipid impurities, carbohydrates or guanidine isothiocyanate residues, hydroxybenzene, polypeptides and suspended conglomerates, as there were no marked peaks in the graphs at wavelengths of 205, 235, 270, 280 and 320 nm, correspondingly. The R_1 extinction coefficient was 1.971 (OD_{262}/OD_{280} = 0.203/0.102) which is close to norm of 2.000 and means almost total absence of polypeptides and residues of carbolic acid in the extract. The R_2 extinction coefficient was 1.990 (OD_{262}/OD_{235} = 0.203/0.102) which is close to 2.000 and accounts for high purity of the extract. When 1% RNA was substituted with carbohydrates, the R_2 coefficient decreased by 0.002, i.e. polysaccharide content in the obtained preparation did not exceed 2.5% and it is admissible [12].

Based on the results of the spectrum analysis of the RNA extract, the following mean values of extinction were obtained: OD_{262} = 0.203 ± 0.002, OD_{320} = 0.002, OD_{260C-} = 0.006 (for OD_{320} and OD_{260}). The average number of RNA molecules isolated from FMDV particles of SAT-2/ Saudi Arabia 7/2000 strain (with 146S sedimentation coefficient) was 54,864,590,497 ± 562,713,752. Concentration of FMDV 146S component was 0.764 ± 0.008 μg/cm^3 which had 98.10 and 99.48% correlation with the results of quantitative CFT (0.75 ± 0.14 μg/cm^3) and rtRT-PCR (0.765 ± 0.044 μg/cm^3), correspondingly. Thus, the previously developed linear mathematical model makes it possible (with the help of a spectrum analysis) to determine concentration of the whole FMDV particles in the non-inactivated suspension for vaccine preparations [8].

At the end of the research, the spectrometric method was tested for its ability to determine concentration of 146S particles in 410 non-inactivated suspensions of FMDV vaccine strains A/Turkey/2006, A/ARRIAH/2015, O/Primorsky/2012, O/Primorsky/2014, Asia-1/Tajikistan/2011, SAT-2/ Saudi Arabia 7/2000. Quantitative rtRT-PCR and CFT tests were simultaneously used in three replications. The actual results obtained in real-time reverse transcription – polymerase chain reaction (rtRT-PCR) were 97.0–99.9% consistent with the expected results of the spectrometric analysis used to determine cultural FMDV 146S component concentration. When compared to a complement fixation test, the actual results were 94.5–99.5% in line with the expected ones. Actual results for positive control were 99.0–99.6% in line with the expected ones. As expected, no FMDV RNA or 146S particles were detected in the negative control sample. Thus, the carried out research demonstrated that the spectrum analysis used to determine concentration of 146S component (when estimating the number of FMDV RNA isolated after serological binding of the whole particles with the help of strain-specific IgG) had 94.5–99.9% correlation with quantitative variants of CFT and rt RT-PCR.

CONCLUSION

We assessed the feasibility of a spectrum analysis to determine indirectly concentration of 146S component based on the number of FMDV RNA molecules isolated after serological binding of the whole virus particles with the help of IgG. This method is cheap, easy-to-use and makes it possible to determine concentration of FMDV 146S particles in non-inactivated suspencion for FMD vaccine within 3–4 hours.

Tests of non-inactivated suspensions of cultural FMDV strains A/Turkey/2006, A/ARRIAH/2015, O/Primorsky/2012, O/Primorsky/2014, Asia-1/Tadjikistan/2011, SAT-2/Saudi Arabia 7/2000 proved that the linear algebraic model C_{146S} = (3.9 × $N_{RNA\ 146S}$ + 566,783,689)/280,818,944,837 makes it possible (with the help of a spectrum analysis) to assess indirectly concentration of FMDV 146S component in the raw materials for a wide range of vaccines.

It was found, that the spectrum analysis used to measure the whole virus particles (when estimating the number of FMDV RNA isolated after serological binding) had 94.5–99.9% correlation with quantitative CFT and rtRT-PCR.

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The use of specialised Sheff-Vax ACF supplements for BHK-21/SUSP/ARRIAH cell cultivation and FMDV reproduction

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SUMMARY

Compliance with the existing purity and safety requirements for immunobiologicals can be effectively achieved by the use of serum-free nutrient media and specialised supplements of non-animal origin. The paper shows the possibility of using Sheff-Vax ACF® supplements (Kerry, Inc., Ireland) for BHK-21/SUSP/ARRIAH cell cultivation and FMDV reproduction. By passage 7, cell concentration and growth rate with Sheff-Vax Plus PF ACF were found to be 40–60% higher than with Sheff-Vax PF ACF and Sheff-Vax Plus ACF. No differences were observed as regards changes in pH. During FMDV reproduction in the cells, it was found that the number of 146+75S components in the test samples containing 1 million cells was 2.3–2.4 higher compared to the controls. Cells cultured with the use of Sheff-Vax Plus PF ACF supplement had normal morphology and multiple dynamic protrusions. In the presence of this supplement, growth rate and suspension concentration in the test and control samples became equal by passage 7. The number of immunogenic components of FMDV reproduced in the cells grown using Sheff-Vax Plus PF ACF was 20–30% higher than in the cells grown using other supplements. BHK-21/SUSP/ARRIAH cell concentration and growth rate in the presence of specialised supplements were found to be lower than those in the control samples with serum and blood protein hydrolysate added to the nutrient medium. The virus yield from 1 million cells was higher in the culture grown using Sheff-Vax ACF supplements. Sheff-Vax Plus PF ACF was found to be the most suitable for BHK-21/SUSP/ARRIAH cell cultivation and FMDV reproduction in the said cells out of the three tested supplements.

Keywords: Sheff-Vax supplements, growth rate, BHK-21/SUSP/ARRIAH cells, foot-and-mouth disease virus (FMDV).

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Использование специализированных добавок Sheff-Vax ACF для культивирования клеток BHK-21/SUSP/ARRIAH и репродукции вируса ящура

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РЕЗЮМЕ

Предъявляемые в настоящее время к иммунобиологическим препаратам требования чистоты и безопасности могут быть эффективно достигнуты при использовании бессывороточных питательных сред и специализированных добавок неживотного происхождения. В данной работе показана возможность применения добавок Sheff-Vax ACF®, производимых компанией Kerry, Inc. (Ирландия), для культивирования культуры клеток ВНК-21/SUSP/ARRIAH и репродукции вируса ящура. Было отмечено, что к седьмому пассажу в присутствии добавки Sheff-Vax Plus PF ACF концентрация клеток и кратность прироста были выше на 40–60%, чем при внесении добавок Sheff-Vax PF ACF и Sheff-Vax Plus ACF. Не обнаружено различий в изменении водородного показателя. При репродукции вируса ящура в полученных клетках определили, что в опытных образцах с 1 млн клеток 146+75S компонентов было больше в 2,3–2,4 раза по сравнению с контролем. При культивировании с добавкой Sheff-Vax Plus PF ACF клетки имели нормальную морфологию, множество динамических выростов. В присутствии данной добавки к седьмому пассажу такие показатели, как кратность прироста и концентрация суспензии, в контрольных и опытных образцах выравнивались. Количество иммуногенных компонентов вируса ящура, репродуцированного в клетках с указанной добавкой, было выше на 20–30%, чем в клетках, выросших с применением других добавок. Установлено, что концентрация клеток линии ВНК-21/SUSP/ARRIAH и кратность прироста в присутствии специализированных добавок была меньше, чем в контрольных образцах с добавлением сыворотки и гидролизата белков крови в питательную среду. При этом выход вируса с 1 млн клеток был выше в культуре, выросшей при внесении добавок Sheff-Vax ACF. Из трех исследованных добавок наиболее приемлемой для культивирования линии ВНК-21/SUSP/ARRIAH и репродукции вируса ящура в полученных клетках была Sheff-Vax Plus PF ACF.

Ключевые слова: Добавки Sheff-Vax, кратность прироста, клетки ВНК-21/SUSP/ARRIAH, вирус ящура.

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INTRODUCTION

A nutrient medium helps maintain cell viability and supports their growth. It serves as a source of nutrients, growth factors and hormones, as well as regulates culture pH and osmotic pressure.

There are several types of media according to whether the growth of cells requires the presence of serum: basal media that require supplementation with 10% serum; advanced media that require supplementation with 1–5% serum; serum-free media that do not require supplementation with serum [1, 2].

The use of blood serum in cell culture has some significant drawbacks. For most tissues, this component is not the body fluid with which they had contact in the original tissue; therefore, serum promotes fibroblast growth, but inhibits the growth of epidermal keratinocytes. Besides, serum can be cytotoxic due to polyamine oxidase that has an effect on polyamines (spermine, spermidine) being the secretory products of rapidly proliferating cells (fetal serum contains relatively high levels of such enzymes). The drawbacks also include a significant serum composition variability in different batches; the amount of specific growth factors in sera can be insufficient, making it necessary to add them to cell cultures. Serum is often contaminated with viruses, many of which, though being not harmful for the cell culture, represent an additional uncontrollable factor [2].

The existing purity and safety requirements for immunobiologicals can be effectively met only by means of serum-free technology. Therefore, intensive studies have been carried out during the past two decades to develop serum-free nutrient media and specialised non-animal supplements, with their formulas being the intellectual property of companies and unavailable for common use. Such media have certain advantages such as improved reproducibility of test results due to the high stability of medium composition; decreased risk of viral, fungal, mycoplasma contamination of cell cultures; facilitation of cell metabolite removal; reduced effect of additional proteins on biological test results; the absence of cytotoxicity [3, 4].

Kerry, Inc. (Ireland) has developed several types of specialised Sheff-Vax ACF® supplements that contain milk product, egg, wheat, peanut derivatives, fish and mollusc products, etc. and differ in mineral and amino acid composition, growth factor concentration and intact protein content.

The aim of the study was to examine the possibility of using specialised serum-free Sheff-Vax ACF supplements for BHK-21/SUSP/ARRIAH cell suspension culture and FMDV reproduction.

MATERIALS AND METHODS

Cell line. A continuous suspension line of neonatal Syrian hamster kidney cells (BHK-21/SUSP/ARRIAH cell line) was used in the study [5].

Specialised supplements. The following non-animal Sheff-Vax ACF supplements (Kerry, Inc., Ireland) were used in the study: Sheff-Vax Plus PF ACF (supplement 1); Sheff-Vax PF ACF (supplement 2); Sheff-Vax Plus ACF (supplement 3).

Specialised supplements at a concentration of 10 g/dm^3 were added to the cell growth medium.

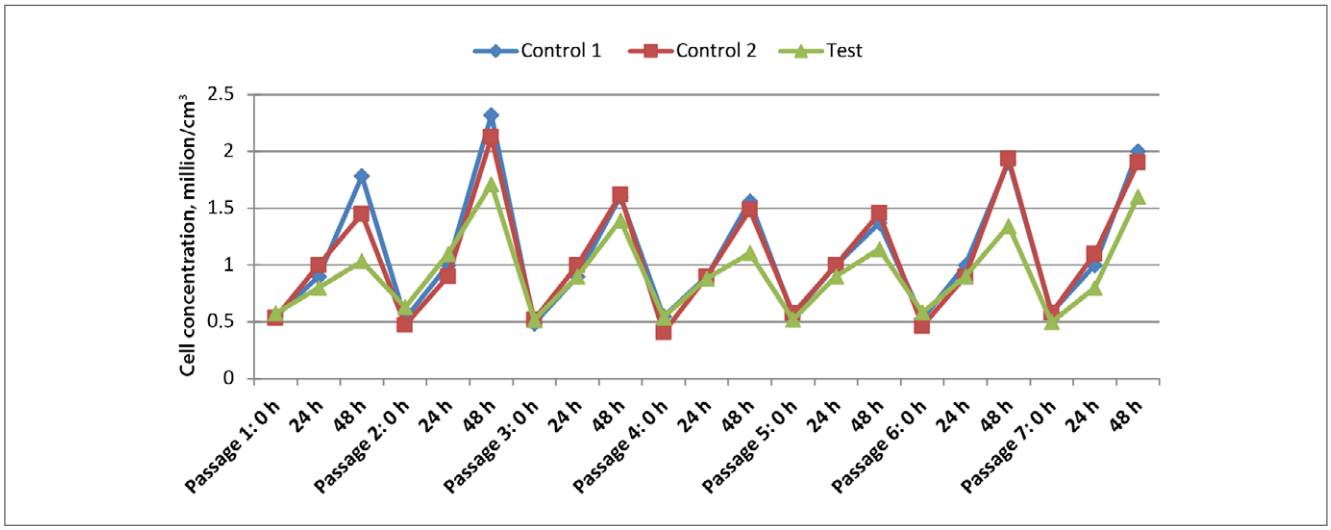

Fig. 1. BHK-21/SUSP/ARRIAH cell concentration dynamics in the presence of specialised Sheff-Vax Plus PF ACF supplement (No. 1)

Рис. 1. Динамика изменения концентрации клеток BHK-21/SUSP/ARRIAH при использовании специализированной добавки Sheff-Vax Plus PF ACF (№ 1)

The nutrient medium used to grow cells was prepared according to the Procedure for production of adsorbed polyvalent and monovalent vaccine against foot-and-mouth disease (based on the virus grown in BHK-21 cells), but no serum was added.

Bovine serum. Fetal bovine serum (Serana, Germany) at a concentration of 5% was used for the tests.

Growth rate was determined as the ratio between the final and initial cell concentrations within one passage (within 48 hours).

Cell line adaptation. At the initial stage of adaptation, cells were collected from suspension contained in the 5% serum-supplemented medium. BHK-21/SUSP/ARRIAH cell line adaptation was carried out during seven successive passages. Sodium hydrogen carbonate solution (7.5%) was used for pH adjustment 24 hours after reseeding.

The following controls were used: control 1 – a medium with constant serum content (5%), control 2 – a medium with serum percentage reduced by half with each successive passage.

A medium containing a specialised Sheff-Vax ACF supplement at a concentration of 10 g/dm^3 was used as a test medium. Serum content in the test medium was also reduced by half with each successive passage.

Viscosity. To achieve the desired viscosity, Pluronic F-68, a polymeric component, at a final concentration of 0.125% was used; it was added to control 2 and test samples from passage 2 on.

Cell infection with FMDV. Culture FMDV Asia-1/Tajikistan/2011 strain at a dose of 1.0 $TCID_{50}$/cell was used to infect suspension BHK-21/SUSP/ARRIAH cells. Passage 7 cell suspension was poured into roller bottles and, where necessary, diluted with the medium to reach a volume of 400–600 cm^3 and a cell concentration of 1.5×10^6 cells/cm^3. FMDV reproduction took place during 16 hours and was followed by the virus inactivation and suspension purification.

Cytochemical study of BHK-21/SUSP/ARRIAH cell morphology was conducted using the luminescence microscope ML-2B. Native preparations were stained with 0.001% acridine orange solution. Photographs were taken using Leica camera and Zeiss, Olympus, Leica microscopes.

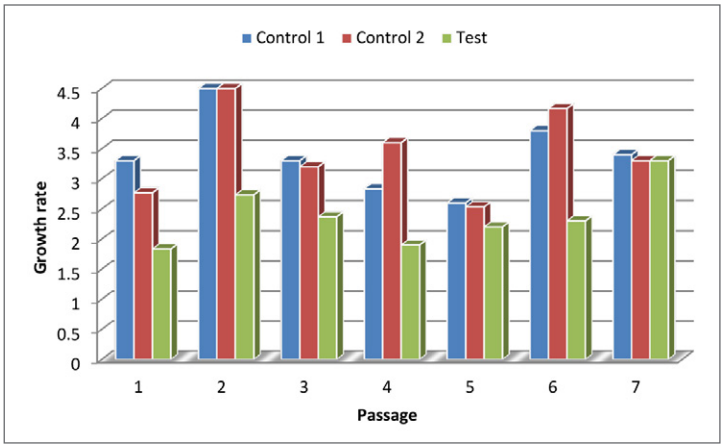

Fig. 2. BHK-21/SUSP/ARRIAH cell growth rate dynamics in the presence of specialised Sheff-Vax Plus PF ACF supplement (No. 1)

Рис. 2. Динамика изменения кратности прироста клеток BHK-21/SUSP/ARRIAH при использовании специализированной добавки Sheff-Vax Plus PF ACF (№ 1)

Viral antigen inactivation and purification. FMDV was inactivated using a 15–20% solution of aminoethylethylenimine . To remove ballast proteins, in particular FMDV non-structural proteins, from the inactivated antigen suspension, a 0.007% solution of Polysept (polyhexamethylene guanidine) was used, with subsequent decanting of supernatant.

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

RESULTS AND DISCUSSION

During the first stage of the study, the dynamics of BHK-21/SUSP/ARRIAH cell concentration was examined

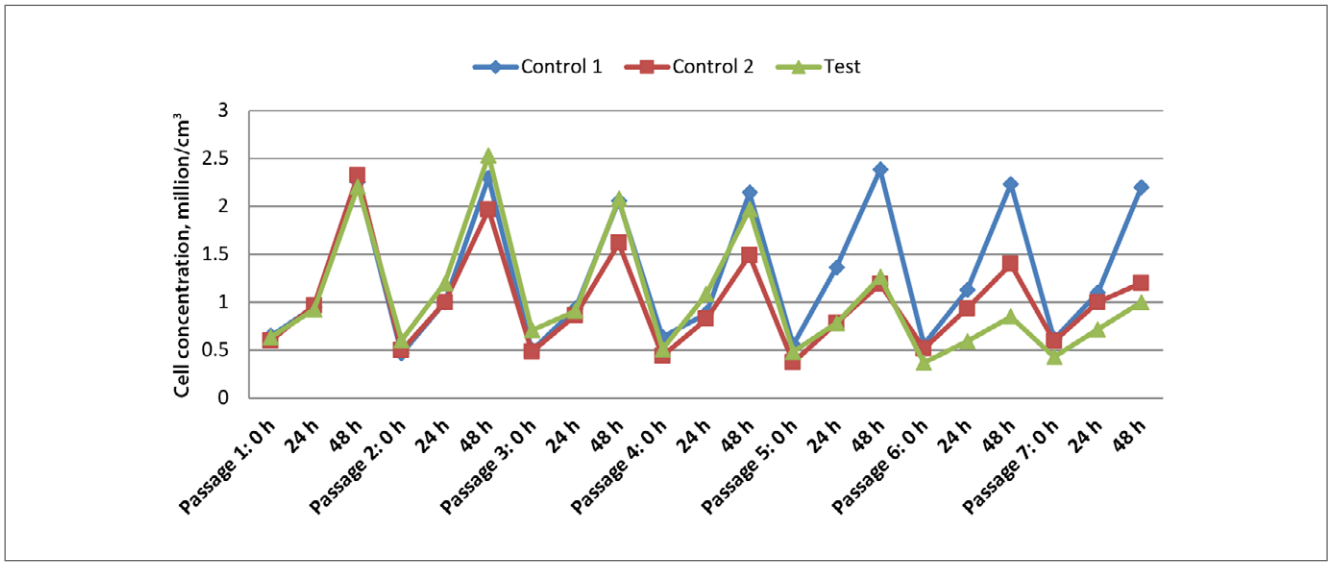

Fig. 3. BHK-21/SUSP/ARRIAH cell concentration dynamics in the presence of specialised Sheff-Vax PF ACF supplement (No. 2)

Рис. 3. Динамика изменений концентрации клеток BHK-21/SUSP/ARRIAH при использовании специализированной добавки Sheff-Vax PF ACF (№ 2)

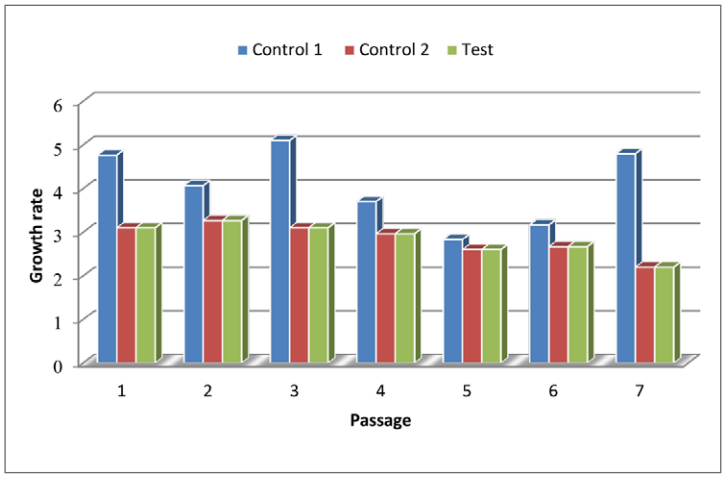

Fig. 4. BHK-21/SUSP/ARRIAH cell growth rate dynamics in the presence of specialised Sheff-Vax PF ACF supplement (No. 2)

Рис. 4. Динамика изменений кратности прироста клеток BHK-21/SUSP/ARRIAH при использовании специализированной добавки Sheff-Vax PF ACF (№ 2)

at different passages in the presence of specialised Sheff-Vax Plus PF ACF supplement (No. 1). The results are presented in Figures 1 and 2.

It was found that cell concentration in the control samples at the end of different passages varied from 1.45 ± 0.04 million/cm^3 to 2.32 ± 0.29 million/cm^3. As for the test samples, cell concentration varied from 1.03 ± 0.03 million/cm^3 at passage 1 to 1.60 ± 0.05 million/cm^3 at passage 7. Cell growth rate in the test samples during the first six passages was 1.18–1.80 times lower than that in the control.

The use of the supplement had no effect on the pH of the medium during cultivation. The pH values changed identically in all the samples, being 6.84–7.11 at the beginning of the passage and decreasing to 6.34–6.48 after 48 hours.

Tests of specialised Sheff-Vax PF ACF supplement (No. 2) showed that cell concentration 48 hours after seeding in the control samples with constant serum content varied from 2.74 ± 0.08 to 1.80 ± 0.10 million/cm^3, and in the last passage 7 it was 2.24 ± 0.18 million/cm^3. Cell growth rates at different passages were in the range of 2.83 to 5.10 (Fig. 3, 4). Cell concentration at the end of passages in the control samples with reduced serum percentage in the nutrient medium and in the test samples with the specialised supplement was the same, but by passage 7 it declined by a factor of 1.4–1.9 when serum level was reduced to 0.075%. Cell growth rates in control 2 and test samples were also the same, namely 2.2–3.1 depending on the passage.

The use of the supplement had no effect on the pH of the medium during cultivation.

Tests of specialised Sheff-Vax Plus ACF supplement (No. 3) showed that cell concentration at passage 7 in the test samples with the medium almost free from serum was 1.0 ± 0.2 million/cm^3, in control 1 – 2.2 ± 0.2 million/cm^3, in control 2 – 1.20 ± 0.06 million/cm^3. Cell growth rate was 2.33 ± 0.03 in the test samples, and 3.57 ± 0.64 and 2.00 ± 0.53 in two control samples (Fig. 5, 6). Cell concentration in the control with constant serum content 48 hours after seeding at different passages was within the range of 2.06–2.38 million/cm^3; cell concentration in the samples with reduced serum content decreased from 2.32 ± 0.18 to 1.20 ± 0.06 million/cm^3 as the passage number increased. Growth rate in the controls with constant serum content was 3.4–5.0; growth rate in the control with reduced serum content was 3.33–4.00 at the first five passages and then decreased to 2.0 by passage 7. Cell growth rate in the test medium containing supplement 3 decreased by a factor of 1.6–1.8 after passage 4.

The use of this supplement had no effect on the pH of the medium during cultivation.

During FMDV reproduction in the cells, it was found that the concentration of 146+75S immunogenic components in the control samples with constant serum content

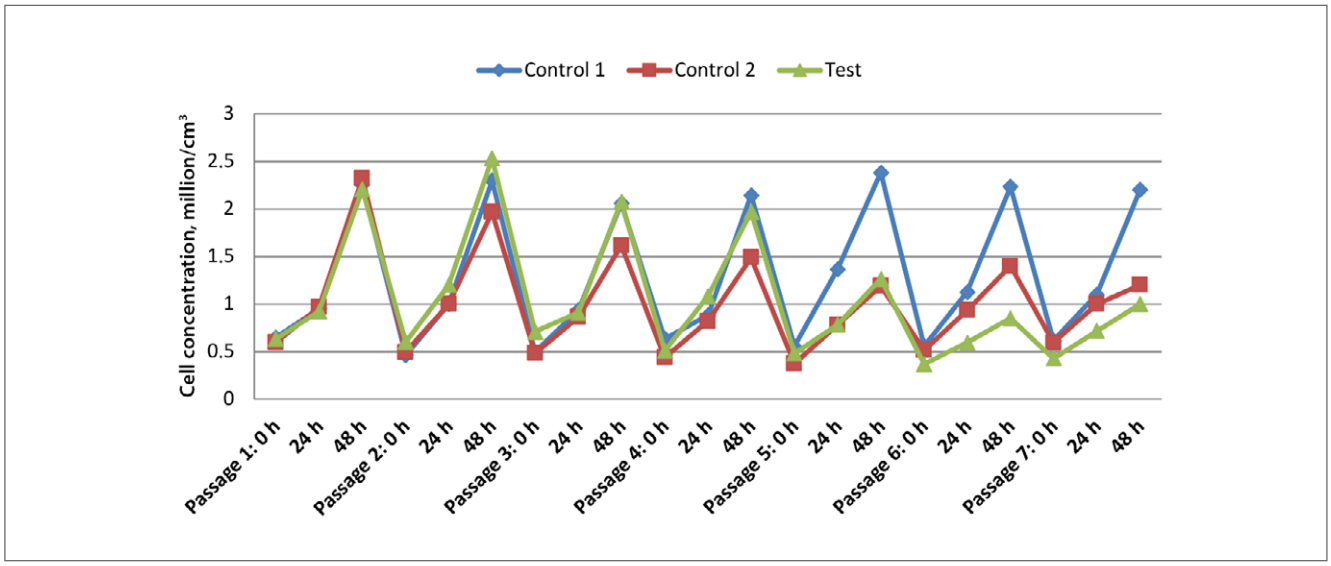

Fig. 5. BHK-21/SUSP/ARRIAH cell concentration dynamics in the presence of specialised Sheff-Vax Plus ACF supplement (No. 3)

Рис. 5. Динамика изменений концентрации клеток BHK-21/SUSP/ARRIAH при использовании специализированной добавки Sheff-Vax Plus ACF (№ 3)

was 1.57, 1.87, 1.60 times lower than in the control samples with reduced serum percentage, and 2.4, 2.3, 2.4 times lower than in the test samples (see Table). Differences were found to be significant ($p < 0.05$).

To study cell morphology, passage 7 suspension was seeded to 50 cm^3 flasks at a concentration of 100 thousand cells/cm^3; cytochemical tests were carried out at passage 8. Cells grown with supplement 1 demonstrated partial adhesion. Sedimented cells had normal morphology and multiple dynamic protrusions indicative of normal physiological activity (Fig. 7).

Partial sedimentation without adhesion was observed in the cells adapted to supplement 2 at passage 8. From the very beginning of cultivation, cell population aggregation was observed that reached its maximum on day 2. No culture proliferation was observed (Fig. 8A).

When supplement 3 was used (Fig. 8B), induced cell aggregation occurred and large colonies (up to 100 cells) were formed. Aggregated cells had irregular spherical shape and showed no signs of trophic activity, i.e. the cells did not divide but merely survived.

The serum-containing control (Fig. 7A) showed 100% confluence; the cells demonstrated 60–80% adhesion and had adaptive traits of a monolayer culture, some of them became spindle-shaped.

CONCLUSION

BHK-21/SUSP/ARRIAH cell line was adapted to specialised Sheff-Vax ACF supplements (Kerry, Inc., Ireland). By passage 7, cell concentration and growth rate in the presence of Sheff-Vax Plus PF ACF (supplement 1) were 40–60% higher than with other supplements. In the presence of supplement 1, cell concentration and growth rate in the test and control samples were equal: 1.6 ± 0.2 million/cm^3, 1.90 ± 0.18 and 2.0 ± 0.2 million/cm^3, respectively; differences were insignificant. Other supplements provided worse performance with respect to cell growth as compared to the control samples – cell concentration and growth rate were 2.0–2.2 times lower.

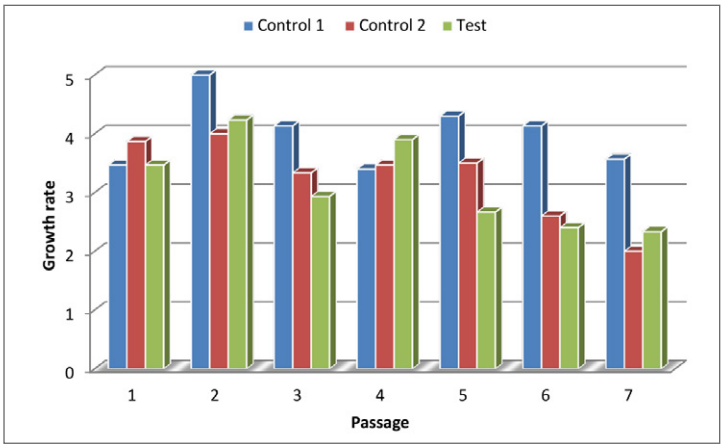

Fig. 6. BHK-21/SUSP/ARRIAH cell growth rate dynamics in the presence of specialised Sheff-Vax Plus ACF supplement (No. 3)

Рис. 6. Динамика изменений кратности прироста клеток BHK-21/SUSP/ARRIAH при использовании специализированной добавки Sheff-Vax Plus ACF (№ 3)

Table
FMDV reproduction in cells grown using specialised Sheff-Vax ACF supplements

Таблица
Репродукция вируса ящура в клетках, выращенных в присутствии специализированных добавок Sheff-Vax ACF

Supplement	146+75S concentration, μg/cm^3 (calculated for 1.0×10^6 cells/cm^3)		
	Control 1	Control 2	Test
Sheff-Vax Plus PF ACF (No. 1)	0.58 ± 0.09	0.91 ± 0.10	1.40 ± 0.10
Sheff-Vax PF ACF (No. 2)	0.47 ± 0.07	0.88 ± 0.08	1.08 ± 0.09
Sheff-Vax Plus ACF (No. 3)	0.50 ± 0.05	0.80 ± 0.08	1.20 ± 0.09
$p < 0.05$			

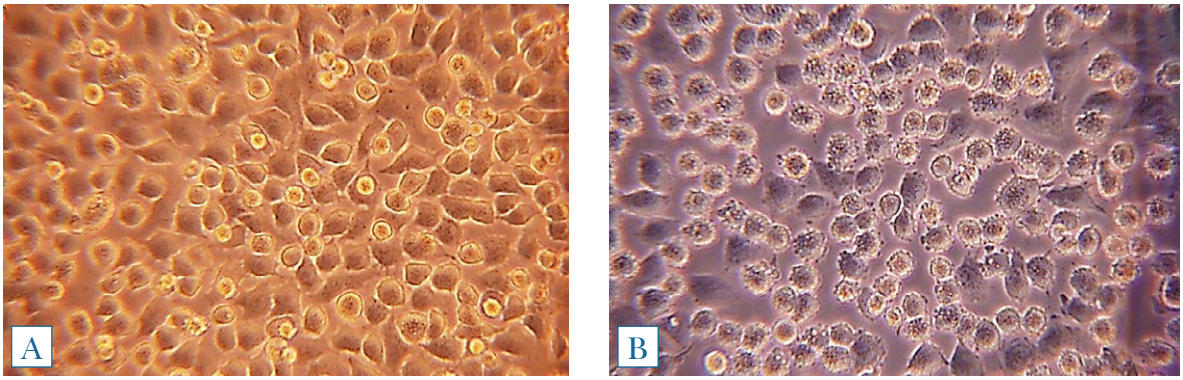

Fig. 7. BHK-21/SUSP/ARRIAH cells adapted to specialised Sheff-Vax Plus PF ACF supplement (No. 1):
A – cells grown in the presence of serum (control),
B – cells grown in the presence of supplement 1 (test)

Рис. 7. Клетки BHK-21/SUSP/ARRIAH, адаптированные к специализированной добавке Sheff-Vax Plus PF ACF (№ 1):
А – клетки, выращенные в присутствии сыворотки (контроль),
В – клетки, выращенные в присутствии добавки № 1 (опыт)

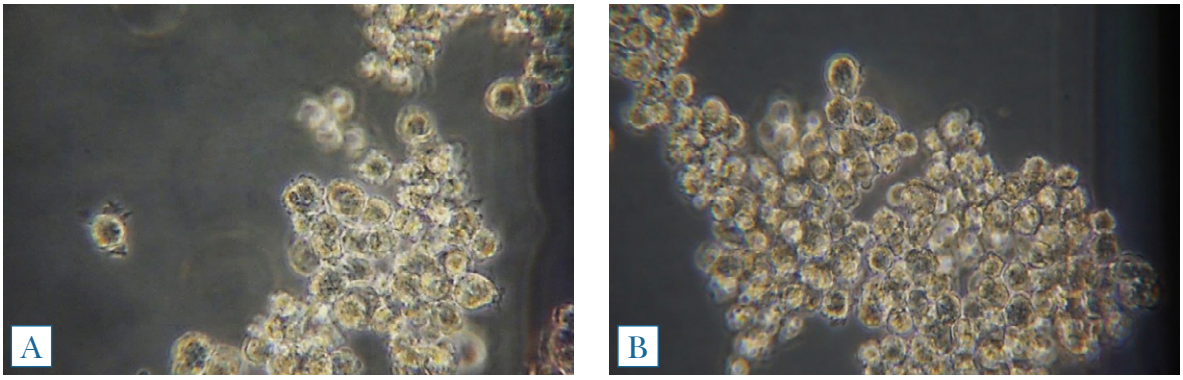

Fig. 8. BHK-21/SUSP/ARRIAH cells adapted to specialised supplements:
A – Sheff-Vax PF ACF (No. 2) and B – Sheff-Vax Plus ACF (No. 3)

Рис. 8. Клетки BHK-21/SUSP/ARRIAH, адаптированные к специализированным добавкам:
А – Sheff-Vax PF ACF (№ 2) и В – Sheff-Vax Plus ACF (№ 3)

No differences were found with respect to changes in pH.

During FMDV reproduction in the said cells, it was found that 146+75S component concentration in the test samples containing 1 million cells was 2.4, 2.3, 2.4 times higher compared to control 1, and 1.54, 1.23, 1.50 times higher compared to control 2 (differences were significant, $p < 0.05$).

Cells grown with the use of Sheff-Vax Plus PF ACF (supplement 1) had normal morphology and multiple dynamic protrusions. By passage 7, cell growth rate and concentration became equal in the suspension of the control and test samples. The number of FMDV immunogenic components in the samples containing supplement 1 was 20–30% higher than in the cells grown with supplements 2 and 3.

Concentration and growth rate of BHK-21/SUSP/ARRIAH cells cultivated with the use of Sheff-Vax ACF supplements were found to be lower than those in the control samples containing serum and blood protein hydrolysate. However, the virus yield from 1 million cells was higher in the cells grown using the specialised supplements.

Thus, serum-free Sheff-Vax ACF supplements (Kerry, Inc.) are suitable for BHK-21/SUSP/ARRIAH cell cultivation and FMDV reproduction. Sheff-Vax Plus PF ACF supplement (No. 1) provided higher results with respect to FMDV immunogenic component concentration.

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Optimization of RHDV type 1 and 2 inactivation modes

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SUMMARY

The purpose of these studies was to optimize RHDV type 1 and 2 (RHDV1 and RHDV2) inactivation modes to use the obtained antigens in inactivated vaccines and diagnosticums. The inactivating effect of aminoethylethylenimine and β-propiolactone was studied in different concentrations in correlation with the exposure time and temperature. The correlation between the inactivating effect of the compound used and the accepted test conditions (concentration, temperature, and exposure time) was studied on a group of rabbits, each of which was injected intramuscularly with 1 cm^3 of the inactivated material sample. At the end of the maximum exposure interval, a control sample of the viral material, kept under the same conditions without any inactivant added was similarly tested. Lethality was considered to evaluate the damaging action in the test and control groups: $L = m/n$, where m is the number of dead animals; n is the total number of rabbits in the group for testing of the inactivated material sample. The post-mortem diagnosis was confirmed by testing the rabbit liver tissue homogenate for relative antigens using ELISA. It was found that aminoethylethylenimine and β-propiolactone did not have the same effect on the studied variants of the virus. In order to preserve at maximum the antigenic structures of the virus, the following inactivation modes were considered to be optimal: for RHDV1 – aminoethylethylenimine at a concentration of 0.3% at 37 °C, exposure time – 72 hours, or β-propiolactone at a concentration of 0.1–0.3% at 25–37 °C, exposure time – 24–48 hours; for RHDV2 – aminoethylethylenimine at a concentration of 1% at 37 °C, exposure time – 72 hours, or β-propiolactone at a concentration 0.3% at 25 °C, exposure time – 24 hours.

Keywords: Viral hemorrhagic disease of rabbits, inactivated vaccine, aminoethylethylenimine, β-propiolactone, inactivation of rabbit hemorrhagic disease virus.

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Отработка режимов инактивации вируса геморрагической болезни кроликов 1-го и 2-го типов

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РЕЗЮМЕ

Цель настоящих исследований состояла в подборе режимов инактивации вируса геморрагической болезни кроликов 1-го (RHDV1) и 2-го (RHDV2) типов для использования полученных антигенов в составе инактивированных вакцин и диагностикумов. Изучали инактивирующее действие аминоэтилэтиленимина и β-пропиолактона в различных концентрациях в зависимости от времени экспозиции и температуры. Оценку инактивирующего эффекта используемого соединения соответственно принятым условиям испытания (концентрация, температура и время экспозиции) проводили на группе

кроликов. Каждому животному делали внутримышечную инъекцию пробы инактивируемого материала в объеме 1 см^3. По истечении максимального интервала экспозиции аналогичным образом испытывали пробу контрольного образца вирусного материала, который содержали при тех же условиях без добавления инактиванта. В опытных и контрольных группах повреждающее действие оценивали с помощью показателя летальности: $L = m/n$, где m – число погибших животных; n – общее количество кроликов в группе для испытания данной пробы инактивируемого материала. Посмертный диагноз подтверждали исследованием гомогената ткани печени кроликов на наличие соответствующих антигенов при помощи иммуноферментного анализа. Установили, что аминоэтилэтиленимин и β-пропиолактон не одинаково воздействовали на исследуемые варианты вируса. В целях максимального сохранения антигенных структур вируса считали, что оптимальными условиями инактивации будут следующие: для RHDV1 – аминоэтилэтиленимином в концентрации 0,3% при 37 °С и экспозиции 72 ч или β-пропиолактоном в концентрации 0,1–0,3% при 25–37 °С и экспозиции 24–48 ч; для RHDV2 – аминоэтилэтиленимином в концентрации 1,0% при 37 °С и экспозиции 72 ч или β-пропиолактон в концентрации 0,3% при 25 °С и экспозиции 24 ч.

Ключевые слова: Вирусная геморрагическая болезнь кроликов, инактивированная вакцина, аминоэтилэтиленимин, β-пропиолактон, инактивация вируса геморрагической болезни кроликов.

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INTRODUCTION

Rabbit hemorrhagic disease (RHD) is an acute, highly contagious disease characterized by hemorrhagic diathesis in all organs, and high mortality (80–100%) [1]. The disease is caused by an RNA virus, a member of the family *Caliciviridae*, genus *Lagovirus*, which is genetically related to the European brown hare syndrome virus [2]. The virions are spherical, 20–40 nm in diameter and have some hemagglutinating properties [3, 4].

RHDV was first recognized in commercially bred Angora rabbits imported from Germany to Jiangsu Province, China, in 1984. Then the disease spread in Europe and Asia [5]. As of 2020, according to the World Organization for Animal Health (OIE), the following countries are recognized as RHD infected: Denmark, Benin, Iceland, Canada, the USA, Mexico, and Finland.

It is believed that weakly virulent variants of the RHDV have been circulating in nature indefinitely. However, in recent decades, the pathogen has undergone significant evolutionary changes, which have resulted in its significantly increased virulence [3].

In 2010, the RHDV type 2 (RHDV2) was isolated in France. The average homology between the genome region encoding the main capsid protein VP60 of the new virus variant and the known strains of this pathogen (RHDV1) was 87%. However, it is important to note that RHDV1 vaccines proved to be ineffective [6].

By 2018, several disease cases associated with RHDV2 were reported in Russia [7]. A characteristic feature that distinguishes the new type virus from RHDV1 is its ability to infect young rabbits under 2 weeks of age [8].

To date, only vaccines containing RHDV1 strains are available in the Russian market of veterinary medicinal products [9]. This fact helped to define the objectives of the study, that is to optimize RHDV type 1 and 2 (RHDV1 and RHDV2) inactivation modes in order to further use the obtained antigens in inactivated vaccines and diagnosticums.

MATERIALS AND METHODS

Strains. The following virus strains were studied: RHDV1 – RHDV1/ARRIAH (infectious titer 3.00 ± 0.25 lg LD_{50}/cm^3); RHDV2 – RHDV2/ARRIAH (infectious titer 4.00 ± 0.25 lg LD_{50}/cm^3).

Virus-containing material. For the studies, a 10% tissue suspension (weight/volume) obtained from the liver tissue of rabbits infected with RHDV1 and RHDV2 was used. The homogenate was prepared in saline solution (0.9% NaCl solution). Dispersion was carried out using a laboratory tissue homogenizer at 10,000 rpm for 10 minutes. The tissue homogenate was stored in the refrigerator at –20 °C. Before use, chloroform (2% by volume) and high-molecular polyhexamethylene guanidine hydrochloride (0.1% by volume) were added to the thawed material, actively mixed and centrifuged at 500 g for 20 minutes. The supernatant was used for further work.

Hemagglutination test. Hemagglutinating activity of the virus was determined in accordance with the techniques set out in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals [1]. The hemagglutinating activity of the prepared virus-containing material was at least 1:1280–1: 2560 HAE.

Enzyme-linked immunosorbent assay (ELISA). RHDV antigen was detected using Ingezim RHDV DAS kit (Ingenasa, Spain) in accordance with the attached instructions [10].

Animals. For the purpose of the study, 360 Soviet chinchilla rabbits (aged 45 days, live weight 1.0–1.5 kg) not vaccinated against RHDV obtained from infectious disease-free farms were used. The animals were kept in groups in isolators equipped with automated drinking and feeding systems.

All tests in animals were carried out in strict compliance with the interstate standards for keeping and care of laboratory animals GOST 33216-2014 and GOST 33215-2014, 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.

Rabbits were euthanized with trichloromethane vapours.

Inactivants. To inactivate virus-containing materials, aminoethylethylenimine was used in the form of a 15% aqueous solution (AEEA, LLC NPP Biochemservice, Russia) and *β*-propiolactone (Acros Organics, USA).

Inactivation. *β*-propiolactone was diluted with a phosphate buffer solution to a concentration of 10% before use. Previously, the pH of the AEEA working solution was adjusted to 8.2–8.3 using acetic acid. The prepared inactivants were added to the virus-containing material to the specified final concentrations. The inactivation procedure was performed at 25 and 37 °C. At specified time intervals, samples were taken to determine the completeness of virus inactivation. The control virus without inactivant was kept under the same conditions. Control virus samples were collected after the longest time exposure.

RESULTS

Inactivating effectiveness of the studied compounds in correlation with their concentration, temperature and exposure time was studied using 360 rabbits. Each experimental group consisted of three animals, each of which was inoculated intramuscularly with 1 cm^3 of the inactivated material sample. After the longest time exposure, the control sample was tested similarly.

The animals were clinically monitored for 10 days. Lethality was registered, and its specificity was confirmed by the following macroscopic findings: foamy/bloody exudate from the nostrils; enlarged liver with dystrophic changes; mottled lungs; kidney abnormalities (color from pale to dark red), kidney petechiation; intestinal hyperemia and hemorrhages, most pronounced at the top of the intestinal folds [11].

The damaging effect in both the experimental and control groups was assessed by the lethality index $L = m/n$, where m is the number of dead animals; n is the total number of rabbits in the group used for the inactivated material sample testing.

The post-mortem diagnosis was further confirmed by testing rabbit liver homogenate for RHDV antigen using ELISA. The completeness of inactivation of the studied materials was confirmed by the absence of RHDV antigen in the organs of the survived rabbits 10 days after their inoculation with the tested inactivated material samples.

Assessment of the inactivation effect of the compounds used are presented in the Table.

The test results showed that:

– the RHDV1 strain turned out to be highly sensitive to *β*-propiolactone. Samples obtained after their exposure to all the tested inactivant concentrations under all the exposure conditions did not contain infective virus ($L = 0$);

– the RHDV1 strain showed a certain sensitivity to AEEA. Complete pathogen inactivation was observed at all temperatures only after the exposure to concentrations above 0.2%;

– the RHDV2 strain demonstrated a certain sensitivity to all the tested concentrations of *β*-propiolactone. However, for complete pathogen inactivation at 25 °C, the concentration of at least 0.3% and the exposure time of at least 24 hours were required. At 37 °C, regardless of the exposure time, all the tested concentrations provided complete inactivation of the pathogen;

– the RHDV2 strain demonstrated a relatively high resistance to inactivation with AEEA. At 25 °C and the inactivant concentrations of up to 1.0%, the pathogen completely retained its biological activity at almost all the time intervals. The exception was the interval of 72 hours, at which the mortality rate was $L = 2/3$ (inactivant concentration of 0.45%). Complete virus inactivation at 25 °C was observed after the exposure to 1.0% inactivant for 72 hours. At 37 °C and AEEA concentration of 1.0% the virus was completely inactivated regardless of the exposure time.

It should be noted that all the control samples of the materials containing viruses of both strains retained their biological activity under all the tested exposure conditions. This rules out the possibility of spontaneous virus inactivation during the experiment.

The results of testing using liver tissue homogenate obtained from dead rabbits of the experimental and control groups showed the presence of the RHDV antigen, which confirmed their specific death. The results of test conducted using homogenated liver tissue of the survived rabbits proved the completeness of virus inactivation.

DISCUSSION

The emergence of a new virus variant with significant antigenic differences is always an important event for this ecological niche. This phenomenon is clearly demonstrated by the epidemic situation in Australia, where RHDV2 was detected in May 2015. Retrospective studies have shown that viruses of this family have a rate of genetic evolution $(2.8–5.4) \times 10^{-3}$ substitutions per year, which occur uniformly in both non-structural and structural protein-coding regions of the genome. However, in RHDV2, changes in the region encoding VP60 (the external antigen of the virus) have been observed with a disproportionately high frequency. It has been suggested that this kind of evolutionary process is going on [12].

It is believed that at present, due to its immunological features, RHDV2 has a large epidemic potential, which is confirmed by data on the displacement of RHDV1 strains that previously circulated in the field in France, Spain and Portugal [13].

It should be noted that RHDV2 does not demonstrate clear advantages in terms of its replication in the macroorganism. The average (geometric mean) viral load in the livers of RHDV2-infected rabbits (3×10^8 capsid copies per mg of tissue) was comparable to that observed in RHDV1-infected rabbit livers (2×10^8 capsid copies per mg of tissue). This fact puts into question the association between the RHDV2 replication rate and its virulence.

As part of the study objectives, we determined the conditions under which *β*-propiolactone and AEEA completely inactivate RHDV types 1 and 2, which allows to obtain antigens that can be used to develop inactivated vaccines.

The immunological properties of the vaccine may fully depend on the choice of inactivation methodology. The main criteria for its effectiveness are complete and irreversibile virus inactivation, as well as the preservation of its original antigenic properties.

Table
Correlation between RHDV1 and RHDV2 inactivation completeness and the tested concentrations, temperature, and exposure time

Таблица
Оценка полноты инактивации RHDV1 и RHDV2 соответственно концентрациям химических соединений, температуре и времени экспозиции

Mortality after the inoculation of the virus suspension containing β-propiolactone							
type of virus	D, %	25 °C			37 °C		
		24 hours	48 hours	72 hours	24 hours	48 hours	72 hours
RHDV1	0.10	0/3*	0/3	0/3	0/3	0/3	0/3
	0.20	0/3	0/3	0/3	0/3	0/3	0/3
	0.30	0/3	0/3	0/3	0/3	0/3	0/3
	control**	3/3	3/3	3/3	3/3	3/3	3/3
RHDV2	0.10	1/3	1/3	1/3	0/3	0/3	0/3
	0.20	1/3	1/3	1/3	0/3	0/3	0/3
	0.30	0/3	0/3	0/3	0/3	0/3	0/3
	control	3/3	3/3	3/3	3/3	3/3	3/3
Mortality in rabbits after their inoculation with the virus suspension containing AEEA							
type of virus	D, %	25 °C			37 °C		
		24 hours	48 hours	72 hours	24 hours	48 hours	72 hours
RHDV1	0.10	3/3	2/3	1/3	3/3	2/3	1/3
	0.20	1/3	1/3	1/3	1/3	1/3	1/3
	0.30	0/3	0/3	0/3	0/3	0/3	0/3
	0.45	0/3	0/3	0/3	0/3	0/3	0/3
	1.00	0/3	0/3	0/3	0/3	0/3	0/3
	control	3/3	3/3	3/3	3/3	3/3	3/3
RHDV2	0.10	3/3	3/3	3/3	3/3	3/3	3/3
	0.20	3/3	3/3	3/3	3/3	3/3	3/3
	0.30	3/3	3/3	3/3	3/3	2/3	1/3
	0.45	3/3	3/3	2/3	3/3	2/3	1/3
	1.00	1/3	1/3	0/3	0/3	0/3	0/3
	control	3/3	3/3	3/3	3/3	3/3	3/3

* Ratio of dead animals to the total number of animals in the group;
** suspension without inactivant; D – inactivant concentration (%).
Conditions under which no lethal effect were observed are highlighted in color.

* Отношение погибших животных к общему количеству в группе;
** суспензия без добавления инактиванта; D – концентрация инактиванта (%).
Цветом выделены условия, при которых летальный эффект отсутствовал.

Of the chemical compounds, two types of inactivants are used most often: reticulating (loosening) and alkylating. The agents that reticulate proteins include aldehydes (formaldehyde, glutaraldehyde, and glycidaldehyde). The alkylating agents that affect the structure of nucleic acids include β-propiolactone, ethylenimine, and others [14].

Under optimal conditions, β-propiolactone inactivates, for example, ND virus without changing its

hemagglutinating, neuraminidase and hemolytic activity significantly. This also applies to the chicken infectious bronchitis virus, which, after the exposure to this compound, retains its antigenic properties at a satisfactory level. However, increased concentration of β-propiolactone can result in an undesirable reaction with viral proteins and, thus, to the decrease of antigenic activity [14].

Compounds that interact with the viral genome are called genotoxic. In aqueous solutions, such inactivants break down to form highly active derivatives having an excess positive charge – the so-called electrophilic group (for example, chloroethylamine). The electrophilic group interacts with the negatively charged (nucleophilic) groups of DNA or RNA molecules to form a stable covalent bond. During replication, such a nucleotide, bound to the inactivant molecules, may not be read or read incorrectly by the polymerase, which blocks its replication or results in lethal mutations [15]. An important fact is that nitrogen-containing heterocyclic compounds inactivate viruses in a first-order reaction, and the inactivation rate as well as the endpoint can be determined with sufficient accuracy. This allows to objectively assess the safety of the final product [16].

Ethylenimine and its N-acetyl derivative inactivate a wide range of viruses belonging to several different families under conditions that do not affect the enzymatic or serological properties of a number of proteins [17]. The results of special studies of the properties of FMDV of various types inactivated with ethylene derivatives showed that aziridine-type preparations minimally altered the protein structures of the virion responsible for antigenicity [18]. The study of the structure of the FMDV antigen after inactivation with ethylenimine showed a high percentage of 140S component corresponding to virions with conserved capsid architecture [19]. When the ILT virus was inactivated with formalin, β-propiolactone, methylenimine, and ethylenimine, it was found that the antigen obtained after the exposure to ethylenimine was the most immunogenic [20].

The effectiveness of the inactivant depends on at least three factors: the specified concentration, temperature, and exposure time. According to the PCR results, the concentration of binary ethylenimine 0.001 M did not affect AIV glycoprotein gene during 8 hours of exposure, while the concentration of 0.01 M changed the structure of this gene after 4 hours [21]. The example with FMDV demonstrates that with the increase of temperature from 25 to 37 °C acetylethylenimine at a concentration of 0.01% accelerated the inactivation process by more than an order of magnitude [22]. The pathogen still remained infective when FMDV suspension was treated with 0.05% acetylethylenimine at 37 °C for 8 hours, while with the increase in the exposure time to 12 hours, the virus was completely inactivated [23].

One of the important advantages of such inactivants as β-propiolactone and ethylenimine is that they are completely hydrolyzed within a few hours to form non-toxic products, and therefore there is no need to neutralize them [14].

It is known that formaldehyde and theotropin (A-24) can be used to inactivate rabbit hemorrhagic disease virus [24, 25]. In some instructions for the use of inactivated vaccines against this disease, formalin is indicated as an inactivant [26, 27].

There are several inactivated vaccines available on the Russian market of veterinary medicinal products for RHD prevention: Tissue inactivated hydroxyaluminium vaccine against "Rabbit Hemorrhagic Disease Virus" (FRCVM, Russia) [26]; "Lapimun Gem-2" (BioTestLab, Ukraine) [27]; "Pestorin" (Bioveta, Czech Republic) [28]; "Rabbivak-V" (LLC "TD "Biagro", Russia) [29]. In addition to that, a vector vaccine ("Nobivac® Myxo-RHD", Intervet International B.V., Netherlands) was registered in 2018. It contains a recombinant myxoma virus with the inserted capsid gene of RHDV [30].

However, only "Lapimun Gem-2" contains RHDV1 and RHDV2 antigens, which is officially declared.

Since the level of cross-protection after the immunization with monovaccines is insufficient, the only appropriate solution is to use a combined vaccine containing antigens of both types of the virus [1]. Thus, it was considered reasonable to develop methods for producing such antigens.

CONCLUSION

The results obtained during the assessment of the inactivating effect of the agents used in the study showed that AEEA and β-propiolactone did not have the same effect on RHDV of types 1 and 2. RHDV1 was significantly more sensitive to the used inactivants. In order to preserve at maximum the antigenic structures of the virus, the following inactivation modes were considered to be optimal:

– for RHDV1 – AEEA at a concentration of 0.3% at 37 °C, exposure time 72 hours or β-propiolactone at a concentration of 0.1–0.3% at 25–37 °C, exposure time 24–48 hours;

– for RHDV2 – AEEA at a concentration of 1% at 37 °C, exposure time 72 hours, or β-propiolactone at a concentration of 0.3% at 25 °C, exposure time 24 hours.

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Colostral immunity as an analytical factor in predicting the development of acute respiratory viral infections in calves

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SUMMARY
To reduce the incidence of acute respiratory viral infections in cattle, routine vaccination of mother cows is carried out. There is a direct dependence of the passive immunity level in calves on the vaccination efficacy in cows. The paper presents the results of a study of colostral immunity in calves and post-vaccination immunity in cows against the agents of acute respiratory viral infections in agricultural facilities located on the territory of the Ural and Volga Federal Districts. In the farms under study ($n = 10$), cattle are vaccinated with inactivated vaccines: "COMBOVAC" and "COMBOVAC-R" (OOO Vetbiokhim, Russia), "HIPRABOVIS® 4" (Laboratorios Hipra, S. A., Spain). The study of postvaccinal immunity level in cows showed that the levels of antibodies to infectious bovine rhinotracheitis virus (5.3–8.0 $\log_2$), bovine viral diarrhea virus (3.5–4.8 $\log_2$), bovine parainfluenza-3 virus (6.8–8.5 $\log_2$) and bovine respiratory syncytial virus (4.2-4.5 $\log_2$) in cattle confer protection. When evaluating the results of serological diagnostics of passive immunity in calves to acute respiratory viral infections, it was found that the level of colostral antibodies in them is lower than the level of post-vaccination antibodies in cows: to infectious bovine rhinotracheitis virus by 34.2–58.8%; to bovine diarrhea virus by 37.5–45.0%; to bovine parainfluenza-3 virus by 14.7–35.4 and to bovine respiratory syncytial virus by 23.5-42.2%. To ensure epizootic favourable situation, it is proposed to adjust the schedules of vaccination against bovine diseases in herds, infected by acute respiratory viral infections for dairy farms under study.

Keywords: Cattle, respiratory viral infections, vaccination, colostral immunity, post-vaccination immunity.

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Колостральный иммунитет как аналитический фактор прогнозирования развития острых респираторных вирусных инфекций у телят

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РЕЗЮМЕ

Для снижения заболеваемости крупного рогатого скота острыми респираторными вирусными инфекциями проводится плановая вакцинация коров-матерей. Существует прямая зависимость уровня пассивного иммунитета у телят от эффективности вакцинопрофилактики коров. В работе представлены результаты исследования напряженности колострального иммунитета у телят и поствакцинального иммунитета у коров против возбудителей острых респираторных вирусных инфекций в сельскохозяйственных организациях, находящихся на территории Уральского и Приволжского федеральных округов. В обследованных хозяйствах ($n = 10$) крупный рогатый скот прививают инактивированными вакцинами: «КОМБОВАК» и «КОМБОВАК-Р» (ООО «Ветбиохим», Россия), «HIPRABOVIS® 4» (Laboratorios Hipra, S. A., Испания). Исследование напряженности поствакцинального иммунитета у коров показало, что уровень антител к возбудителям инфекционного ринотрахеита (5,3–8,0 $\log_2$), вирусной диареи (3,5–4,8 $\log_2$), парагриппа-3 (6,8–8,5 $\log_2$), респираторно-синцитиальной инфекции (4,2–4,5 $\log_2$) крупного рогатого скота соответствует протективному. При оценке результатов серодиагностики пассивного иммунитета у телят к острым респираторным вирусным инфекциям установлено, что уровень колостральных антител у них ниже, чем уровень поствакцинальных антител у коров: к вирусу инфекционного ринотрахеита на 34,2–58,8%; к вирусу диареи на 37,5–45,0%; к вирусу парагриппа-3 на 14,7–35,4%; к респираторно-синцитиальному вирусу на 23,5–42,2%. Для обеспечения эпизоотического благополучия предложено провести коррекцию программ специфической профилактики заболеваний крупного рогатого скота в неблагополучных по острым респираторным вирусным инфекциям стадах для подвергнутых обследованию молочно-товарных ферм.

Ключевые слова: Крупный рогатый скот, острые респираторные вирусные инфекции, вакцинация, колостральный иммунитет, поствакцинальный иммунитет.

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INTRODUCTION

Vaccination plays a key role in protecting of cattle from acute respiratory viral infections (ARVIs) and bovine health improving [1]. The main result of systematic and active vaccination is the development of herd specific immunity, which reduces the incidence and, as a rule, reduces the circulation of ARVI agents in the herd [2, 3].

The basis of herd specific immunity is the percentage of individuals that are immune to a particular infection [4]. This takes into account individuals with both active immunity, achieved by vaccination, and with passive immunity, which is formed as a result of the introduction of specific antibodies and/or transfer of immunocompetent cells. The level of specific immunity and duration of protective immunity (the immunological memory phenomenon) depend on the proportion of immune individuals in the population) [4, 5]. The structure of the proportion of immune individuals in different age groups of cattle varies significantly. For example, in one-month-old calves, passive immunity prevails, and the active immunity is predominant in dairy cows. Numerous studies have shown a direct dependence of passive immunity level in calves on the effectiveness of ARVI vaccination in maternal cows [6–9].

Colostral immunity in calves is the main factor that inhibits ARVI virus penetration into cells and their replication before active vaccination programs are started to be implemented. As a rule, a low level of antibodies or their decrease causes the rise in the incidence of respiratory viral infections in young animals. It is important to choose the right time for vaccination, so that, on the one hand, no neutralization of vaccine antigens by colostral immunoglobulins could occur in calves, and on the other – to form a long-term strong immunity in a timely manner. In each herd, this period will depend on many factors, including the level of colostral antibodies in calves (which is associated with the post-vaccination immunity of mother cows, as well as the colostrum feeding technology) and their half-life.

The aim of the study was to analyze the presence of passive antibodies in young cattle compared to the post-vaccination immunity of mother cows in order to predict the onset of ARVI manifestation and plan the vaccination schedule.

MATERIALS AND METHODS

The research was carried out in the Department of Monitoring and Prediction of Infectious Diseases of the Federal State Budgetary Institution, the Ural Federal Agrarian Research Centre, the Ural Branch of the Russian Academy of Sciences (Yekaterinburg) within the framework of Category 160 of the Program of Fundamental Scientific Research by State Academies of Sciences – "Molecular-biological and nanobiotechnological methods of developing new-generation biological products, technologies and methods of their application to combat highly dangerous infectious, parasitic and non-infectious animal diseases (2013–2020)".

The object of the study was cattle kept in factory farms; biological material used was serum of cows and 3–7 day old calves ($n = 327$). Clinical samples of sera were obtained from 10 agricultural facilities located on the territory of the Ural and Volga Federal Districts. In the surveyed farms, cattle are vaccinated with inactivated vaccines: "COMBOVAC" and "COMBOVAC-R" (OOO Vetbiokhim, Russia), "HIPRABOVIS® 4" (Laboratorios Hipra, S. A., Spain).

Serological studies of sera to detect antibodies to the causative agents of infectious bovine rhinotracheitis (IBR), bovine viral diarrhea (BVD), parainfluenza-3 (BPI-3), bovine respiratory syncytial infection (BRS) were performed by indirect hemagglutination (IHA) and hemagglutination inhibition (HI) tests using domestic commercial RBC test kits. The titer of the detected antibodies was expressed as $\log_2$.

RESULTS AND DISCUSSION

The results of serological studies of sera collected from calves during their first month of life and cows are shown in Figures 1–4.

The analysis of the data obtained by the study of post-vaccination immunity in cows showed that the level of antibodies to IBR, BVD, BPI-3 and BRS viruses corresponds to the protective level. The maximum titer of postvaccinal antibodies to IBR virus (8.0 $\log_2$) was found in animals of dairy farms in the Chelyabinsk Oblast, the minimum (5.3 $\log_2$) in cows of dairy farms in the Perm Krai. The level of postvaccinal antibodies to BVD virus in the examined cows ranged from 3.5 $\log_2$ (Kurgan Oblast) to 4.8 $\log_2$ (Udmurt Republic); to BRSV – from 4.2 $\log_2$ (Chelyabinsk Oblast) to 4.5 $\log_2$ (Perm Krai); to the BPIV-3 – from 6.8 $\log_2$ (Udmurt Republic) to 8.5 $\log_2$ (Kurgan Oblast).

When analyzing the results of serological diagnostics of passive immunity to ARVIs in calves, it was found that in some cases the level of colostral antibodies was lower than the level of post-vaccination antibodies in cows.

For example, in calves of dairy farms of the Kurgan Oblast, the level of colostral antibodies to BVDV was lower by 7.14%, to the BPIV-3 by 14.70%, to the BRSV – by 23.52%. In calves of dairy farms in the Chelyabinsk Oblast, a decrease in colostral antibodies to IBR virus was registered by 58.75%, to BVD virus – by 42.50%, to BPIV-3 – by 35.36% and to BRS virus – by 38.09%. A similar pattern of colostral antibody deficiency was observed in calves of dairy farms in the Perm Krai: IBRV – by 39.62%, BVD virus – by 45.00%, BPIV-3 – by 25.35%, and to BRS virus – by 42.22%. In calves of dairy farms of the Udmurt Republic, a decrease in the

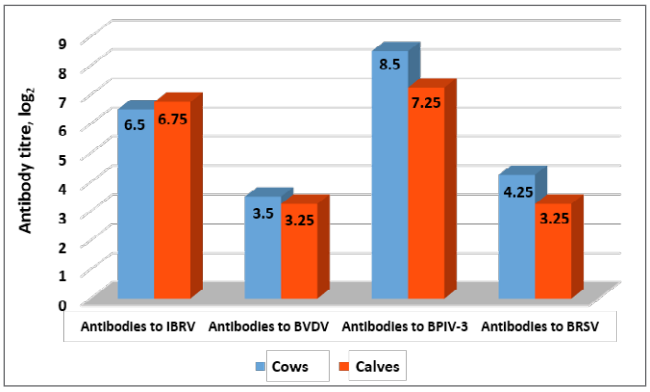

Fig. 1. The level of postvaccinal and colostral antibodies to ARVI agents in animals of dairy farms in the Kurgan Oblast

Рис. 1. Уровень поствакцинальных и колостральных антител к возбудителям ОРВИ у животных молочно-товарных ферм Курганской области

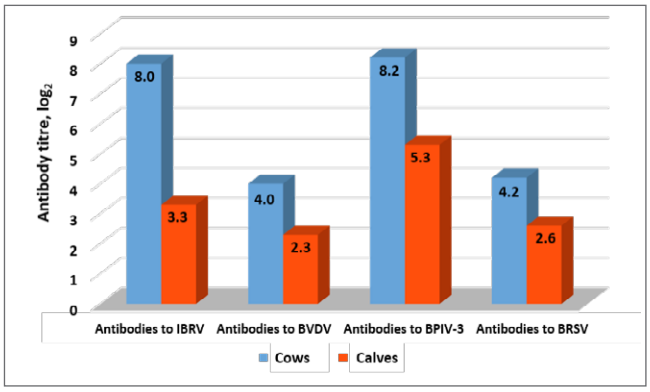

Fig. 2. The level of postvaccinal and colostral antibodies to ARVI agents in animals of dairy farms in the Chelyabinsk Oblast

Рис. 2. Уровень поствакцинальных и колостральных антител к возбудителям ОРВИ у животных молочно-товарных ферм Челябинской области

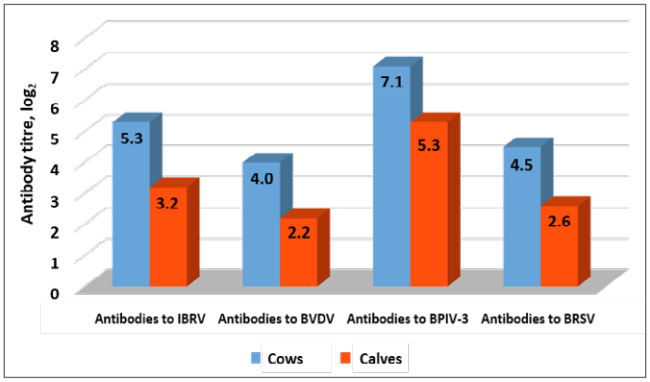

Fig. 3. The level of postvaccinal and colostral antibodies to ARVI agents in animals of dairy farms in the Perm Krai

Рис. 3. Уровень поствакцинальных и колостральных антител к возбудителям ОРВИ у животных молочно-товарных ферм Пермского края

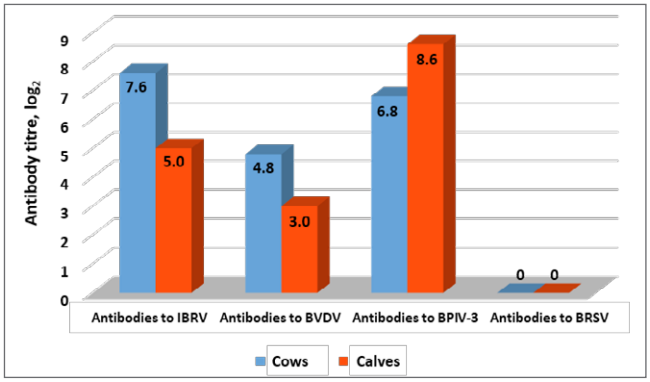

Fig. 4. The level of postvaccinal and colostral antibodies to ARVI agents in animals of dairy farms in the Udmurt Republic

Рис. 4. Уровень поствакцинальных и колостральных антител к возбудителям ОРВИ у животных молочно-товарных ферм Удмуртской Республики

level of colostral antibodies against IBRV by 34.21% and BVDV by 37.50% was established.

The main reasons for the revealed differences in the levels of colostral immunity to ARVI agents in calves and post-vaccination immunity in cows, in our opinion, may be violations of the colostrum drinking technology and/or the vaccination program in mother cows.

CONCLUSION

The results of the study showed that calves on farms in the Kurgan Oblast and Udmurt Republic had a considerably high titer of colostral antibodies compared to the level of post-vaccination antibodies to ARVI pathogens in mother cows. Therefore, the timing of the first vaccination of young animals in these herds can be shifted (taking into account the management practice) to 30–45 days of age. In the herds of the Perm Krai and the Chelyabinsk Oblast, the opposite situation is observed – there is a low transmission of immunoglobulins with colostrum to newborns: by the age of one month, the level of antibodies in the tested calves was at the minimum protective level or even lower. Vaccination of young animals in such herds should be started 1–2 weeks earlier, because a drop in the level of colostral antibodies can lead to an early incidence of ARVIs.

Thus, the determination of colostral antibody level can be used to predict the age when young cattle starts to be affected with respiratory virus infections, and to improve the technology of colostrum feeding, as well as to adjust vaccination schedules to prevent acute respiratory viral infections in cattle herds.

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Problems and prospects of bovine tuberculosis serological diagnosis

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SUMMARY
For the purposes of tuberculosis eradication on any tuberculosis-infected farm, it is necessary to identify tuberculin anergic animals, being a potential source of the infection. The purpose of this study was to analyze the role of complement fixing and haemagglutinating antibodies for the detection cattle infected with bovine tuberculosis (TB). 977 cattle of different sex and age groups on two tuberculosis-infected farms were tested thrice over time. After 35 days all tuberculin reactive cattle (132 animals; 13.5%) were subjected to complex testing using allergy and serology methods. After 40 days (Stage 3), animals demonstrating apparent specific antibody activity and low cell immunity were tested. Allergy tests were proved to be non-informative to diagnose tuberculosis on infected farms. Complement fixing and haemagglutinating antibodies were found to be active in tuberculin anergic animals. A higher antigenicity of Ukrainian RIEVM TB antigen complex as compared to Siberian RVI one was revealed by complement fixation test as well as by indirect haemagglutination test using VIEV polysaccharide antigen; the detection rate was 68 (7.0%), 28 (2.9%) and 299 (30.6%) respectively. The correlation between seropositivity and immunoreactivity was not established. Animals, positive in complement fixation and indirect haemagglutination tests, did not react to tuberculin. Nineteen out of twenty tuberculin reactive animals showed post-mortem lesions, consistent with their seropositivity during post-mortem inspection; moreover, the post-mortem lesions of animals, positive in complement fixation test using Siberian RVI antigen, were consistent in all cases. The results obtained suggest a high performance of allergy test and serological test combination and a promising potential of their complex use for tuberculosis diagnosis in cattle.

Keywords: Tuberculosis, serological tests, allergen, anergy, antibodies, cattle, allergy test, post-mortem tests.

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Проблемы и перспективы серологической диагностики туберкулеза крупного рогатого скота

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РЕЗЮМЕ
При оздоровлении каждого неблагополучного по туберкулезу хозяйствующего субъекта необходимо выявлять анергичных к туберкулину животных, являющихся потенциальным источником инфекции. Целью настоящего исследования было изучение роли комплементсвязывающих и гемагглютинирующих антител при выявлении больного туберкулезом крупного рогатого скота. В двух неблагополучных по туберкулезу хозяйствах исследовано трехкратно в динамике 977 голов скота разных половозрастных групп. Всех реагирующих на туберкулин животных (132 головы; 13,5%) через 35 дней подвергли комплексному исследованию с использованием аллергических и серологических методов. На третьем этапе с интервалом 40 дней исследовали животных с выраженной специфической активностью антител и низким функциональным состоянием клеточного иммунитета. Показана низкая информативность аллергических методов диагностики туберкулеза в неблагополучных по заболеванию хозяйствах. Установлена активность комплементсвязывающих и гемагглютинирующих антител в организме анергичных к туберкулину животных. Более высокая антигенная активность выявлена в реакции связывания комплемента у комплексного туберкулезного антигена Украинского НИИЭВ в сравнении с антигеном Сибирского НИВИ, а также в реакции непрямой гемагглютинации с полисахаридным антигеном ВИЭВ, показатель выявляемости при этом составил 68 (7,0%), 28 (2,9%) и 299 (30,6%) случаев соответственно. Коррелятивной связи между серопозитивностью и иммунореактивностью обнаружить не удалось. Животные с положительными показаниями в реакции связывания комплемента и реакции непрямой гемагглютинации не реагировали на туберкулин. При послеубойном осмотре

у 19 из 20 не реагировавших на туберкулин коров патолого-анатомические изменения совпадали с серопозитивностью, причем у животных с положительными показаниями в реакции связывания комплемента с антигеном Сибирского НИВИ – во всех случаях. Полученные результаты свидетельствуют о высокой результативности комбинации аллергического теста с серологическими методами и перспективности их сочетанного использования для диагностики туберкулеза крупного рогатого скота.

Ключевые слова: Туберкулез, серологические реакции, аллерген, анергия, антитела, крупный рогатый скот, аллергический метод, патолого-анатомические исследования.

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INTRODUCTION

In tuberculosis-infected farms, diseased animals may develop anergy to tuberculin. Such animals do not respond to tuberculin intradermal administration and are a source of infection [1–4].

In the literature, there is enough information about the detection of tuberculin anergic animals using serological tests. Thus, studies of bovine sera by complement fixation test, conducted by I. A. Karkadinovskaya, show the presence of 19.2% of such animals in the herd [5]; according to the studies by E. D. Lakman – from 2 to 3% [6]; V. I. Rotov et al. detected 12% of such animals [7], Yu. Ya. Kassich et al. – 1.5–7.8% [8]. To clarify the results of the allergic test and to detect anergic animals, the complement fixation test (CFT) and the indirect hemagglutination test (IHA test) are mainly used [9].

A. S. Donchenko et al. when studying the specificity of various antigens by CFT on TB free farms, found that in comparison with the phenolic antigen of the Siberian Research Veterinary Institute (Siberian RVI), the tuberculosis antigen complex of the Ukrainian Research Institute of Experimental Veterinary Medicine (Ukrainian RIEVM) had a higher specificity [10].

Indirect hemagglutination test for the diagnosis of TB was studied by such researchers as E. I. Buryak [11], A. I. Kuzin and N. P. Ovdienko [9], V. L. Solodovnikov [12] and others. N. P. Ovdienko et al. established the possibility of application of IHA test using polysaccharide antigen of the All-Union Institute of Experimental Veterinary Medicine (VIEV) for the detection of TB-infected animals as well as tuberculin anergic animals [13].

When conducting CFT and IHA tests, the researchers used a variety of antigens for *in vivo* diagnosis of tuberculosis on farms with different TB epidemic situations, but the results were contradictory.

The aim of the work was to evaluate the effectiveness of serological tests in detecting of cattle anergic to tuberculin on TB-infected farms.

MATERIALS AND METHODS

The practical significance of serological tests was determined on two farms, where the animal health situation was improved by systematic diagnostic studies and removal of reacting animals. In total, 977 cattle of different sex and age groups were tested: on the first farm – 563 animals, of which 269 cows aged 3–4 years and young animals of 1.5–2 years of age (294 animals); on the second farm – 414 animals (219 and 195 animals, respectively).

After 35 days, animals reacting to tuberculin were subjected to a complex study using intradermal, palpebral and ocular tests as well as serological tests.

The studies were conducted in accordance with the "Manual on the diagnosis of animal tuberculosis" (approved by the Veterinary Department of the RF Ministry of Health on 18.11.2002), mammalian tuberculin was administered in the amount of 10,000 international units.

The complement fixation test was performed using Ukrainian RIEVM complex tuberculosis antigen and Siberian RVI phenolic antigen according to the approved methods, and the indirect hemagglutination test using VIEV polysaccharide antigen was carried out by the method of M. Lorid.

Animals that did not respond to tuberculin administration and showing positive results in complement fixation and indirect hemagglutination tests were re-tested using skin test in 40 days. 20 cows, anergic to tuberculin and positive in both tests, were autopsied. The internal organs and lymph nodes were examined and, regardless of the presence or absence of tuberculous changes, the samples were taken for laboratory testing according to the generally accepted method.

RESULTS AND DISCUSSION

As a result of allergic studies, it was found that 132 (13.5%) of 977 animals reacted to tuberculin administration.

When conducting a second complex study after 35 days, various allergic and serological methods revealed: 27 (2.8%) animals responding to a skin test; 24 (2.5%) – to a palpebral test; 4 (0.4%) – to an eye test (Table 1). All animals positive in the eye test, responded in parallel to skin and palpebral tests. Only 5 animals were positive in a palpebral test and 8 animals were positive in a skin test.

Diagnostic titers of complement fixing antibodies in the complement fixation test using Ukrainian RIEVM complex TB antigen were established in 68 (7.0%) cases,

and using Siberian RVI phenolic antigen in 28 (2.9%) cases. When testing sera by indirect hemagglutination test using VIEV polysaccharide antigen, the hemagglutinating antibodies were detected in 299 (30.6%) animals.

Positive results in the complement fixation test were established more often in sera of cows than of heifers. Thus using Ukrainian RIEVM complex TB antigen, diagnostic antibody titers were established on average in 10.5% of cows and in 3.5% of heifers, and using Siberian RVI antigen in 4.5 and 1.2%, respectively. Of the 27 animals with positive skin reactions, the results of complement fixation test using Ukrainian RIEVM complex TB antigen coincided in 3 cases, and using Siberian RVI antigen in 2 cases, while the results of indirect hemagglutination test showed the coincidence in 10 cases. The serological testing of sera by complement fixation test using both antigens demonstrated coincidence of positive results in 26 animals. The results obtained by CFT and IHA test using Siberian RVI antigen coincided in 22 cases, and using Ukrainian RIEVM complex TB antigen in 41 cases.

Skin test-positive animals were isolated. The rest 950 animals with positive results in palpebral and ocular tests, CFT and IHA test were left in herds. After 40 days, they were re-tested by skin test, which revealed 18 (1.9%) reacting cows: 9 out of 471 (1.9%) cows and 9 out of 479 (1.9%) heifers. Of the 96 animals with complement-fixing antibodies in serum, none reacted to the skin test, and of the 299 animals with hemagglutinating antibodies in blood, 10 animals reacted.

A comparative analysis of allergic and serological test results showed no correlation between seropositivity and immunoreactivity. From the animals with positive results in CFT using Ukranian RIEVM complex TB antigen (65 animals), Siberian RVI antigen (26 animals) and in IHA test (299 animals), no tuberculin reacting animals were detected.

In order to compare the positive results of CFT and IHA test with post-mortem lesions in organs, 20 tuberculin anergic cows were killed (Table 2).

In 19 of 20 animals, diagnostic antibody titers were detected by CFT using Ukrainian RIEVM antigen, and in all animals by CFT using Siberian RVI antigen and in 16 animals by IHA test using VIEV polysaccharide antigen. Post-mortem examination revealed TB-characteristic post-mortem lesions in 19 of 20 animals; moreover "pearl disease" was found in 3 animals, a generalized form was revealed in 4 animals, and the remaining 12 animals showed local changes in organs and lymph nodes.

Autopsy revealed no TB-characteristic lesions in one animal with high diagnostic antibody titers in tests using all studied antigens; the diagnosis was established by the bacteriological method. The sera of 14 animals showed positive results in tests using all antigens, each of them had TB consistent changes.

Of the 20 animals killed, showing positive results in CFT using Siberian RVI antigen, tuberculosis was diagnosed in 100% of cases. No antibodies were detected in one animal, showing TB typical changes, by CFT using Ukrainian RIEVM antigen. Also, 4 animals with established tuberculosis, showed negative results in IHA test using VIEV polysaccharide antigen. It was not possible to kill the remaining animals with positive serological tests; tuberculin reacting animals were not killed as well.

The direct correlation between antibody titers and TB severity was not established. The post-mortem examination revealed apparent tuberculosis in animals with both high and low antibody titers. Thus, in 3 animals, showing titers not exceeding 1:20 and 1:10 in CFT using Ukrainian RIEVM and Siberian RVI antigens, respectively, generalized tuberculosis was detected. A similar pattern was observed in one animal, showing negative results in IHA test.

Thus, the use of tuberculin skin test alone for TB diagnosis in infected herds does not allow to identify all diseased animals, which suggests the need for complex studies.

It should be noted that most researchers have studied various serological methods, including CFT and IHA test for the diagnosis of bovine tuberculosis in sera from tuberculin-responsive animals. Herewith, the lack of their effectiveness was noted. It is known that the tuberculin test and serological methods reveal different stages of the infectious process.

Table 1
Results of animal complex testing for tuberculosis

Таблица 1
Результаты комплексного исследования животных на туберкулез

Farm number	Sex-age group of animals	Number of animals	The number of tuberculin reacting animals			Antibody diagnostic titers		
			skin test	palpebral test	eye test	CFT (Ukranian RIEVM antigen)	CFT (Siberian RVI)	IHA test (VIEV antigen)
1	Cows	269	9 (3.3)*	6 (2.2)	2 (0.7)	46 (17.1)	21 (7.8)	96 (35.7)
	Heifers	294	3 (1.0)	4 (1.4)	–	9 (3.0)	1 (0.3)	57 (19.4)
2	Cows	219	6 (2.7)	6 (2.7)	–	5 (2.3)	1 (0.5)	48 (21.9)
	Heifers	195	9 (4.6)	8 (4.1)	2 (1.0)	8 (4.1)	5 (2.6)	98 (50.3)
Total		977	27 (2.8)	24 (2.5)	4 (0.4)	68 (7.0)	28 (2.9)	299 (30.6)

CFT – Complement fixation test (РСК – реакция связывания комплемента);

IHA test – Indirect haemagglutination test (РНГА – реакция непрямой гемагглютинации);

* Percentage is indicated in parenthesis (в скобках указаны проценты).

Table 2
Results of serological and post-mortem studies

Таблица 2
Результаты серологических и патолого-анатомических исследований

Animal number	Antibody titer			TB consistent lesions
	CFT (Ukranian RIEVM antigen)	CFT (Siberian RVI antigen)	IHA test (VIEV antigen)	
1	1:40	1:40	1:64	in the mediastinal lymph nodes
2	1:20	1:10	1:32	in the pharyngeal, portal lymph nodes, liver
3	1:20	1:40	1:64	in the pharyngeal, mediastinal, bronchial lymph nodes
4	1:20	1:10	1:64	generalized tuberculosis
5	1:160	1:40	1:128	"pearl disease"
6	1:80	1:20	1:32	in the parotid lymph nodes, pulmonary tuberculosis
7	1:160	1:40	1:16	"pearl disease"
8	1:20	1:20	1:16	"pearl disease"
9	–	1:10	1:16	bronchial tuberculosis, pulmonary tuberculosis
10	1:320	1:40	1:16	not found (tuberculosis / diagnosis established by bacteriological method)
11	1:20	1:40	1:16	in the pharyngeal lymph nodes
12	1:40	1:10	1:16	in the bronchial lymph nodes
13	1:40	1:10	1:16	in the bronchial lymph nodes
14	1:20	1:10	–	in the bronchial lymph nodes, lungs, udder
15	1:160	1:10	–	in the lungs
16	1:80	1:40	1:128	generalized tuberculosis
17	1:20	1:10	1:64	generalized tuberculosis
18	1:20	1:10	–	generalized tuberculosis
19	1:80	1:10	–	in the liver
20	1:80	1:10	1:128	in the pharyngeal, submandibular lymph nodes, liver, lungs

The data obtained in the course of this study suggest a possible blockade of the immunity T-system and, as a result, the malfunctioning of the antigen-dependent lymphocyte differentiation in response to mycobacteria activitiy. The results coincide with the data of other authors, suggesting that T-system blockade occurs in some diseased animals, followed by temporary or permanent lack of allergic reactions while humoral antibodies are maintained.

The decrease in the cellular immunity functioning of individual TB-infected animals requires a more extensive research.

CONCLUSION

In herds infected with TB for longer periods, CFT using TB antigens and IHA test using VIEV polysaccharide antigen allows additional detection of diseased animals that are anergic to tuberculin. On these farms, it is advisable to perform complex diagnostic tests using serological methods.

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Role of bovine respiratory syncytial virus in etiology of respiratory diseases on milk farms

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SUMMARY
Bovine respiratory syncytial virus (BRSV) is one of the etiological agents of respiratory diseases. The agent spreads widely in all the countries with intensive livestock farming and can cause pathologic changes in respiratory system either alone or in combination with other viruses and bacteria. It is a matter of crucial importance to study spread of the agent on large milk farms, to detect it in the internal organs of infected animals, and to quantify virus accumulation in them. The purpose of the research was to study peculiarities of RS infection spread, frequency of the virus detection in biomaterial samples (both alone and in associations with infectious bovine rhinotracheitis (IBR) and bovine viral diarrhea/mucosal disease viruses (BVDV) and with *Pasteurellaceae* bacteria) on large milk farms affected by respiratory animal diseases; and to determine virus concentration in the respiratory organs. BRSV alone was reported in 9.2% of the tested biomaterial samples, as associated with IBR and BVDV it was reported in 1.4% and 5.2% of samples, correspondingly. The number of samples containing simultaneously BRSV and *Pasteurellaceae* bacteria was 10.8%. The virus was reported in a maximum of 26.6% of the tested samples. With the help of real-time PCR the virus genome was detected in lungs (13.1%), in exudate from trachea, bronchi and nasal sinuses (6.0%), in nasal discharge (4.0%) and in bronchi (1.7%). The virus was seldom detected in trachea and bronchial mucosa (1.1%) and in pulmonary lymph nodes (0.8%). Quantification of BRSV RNA demonstrated that maximum virus accumulation was observed in lungs and nasal charges and it confirms data on its tropism to pulmonary interstitium.

Keywords: Cattle, respiratory diseases, respiratory syncytial virus (RSV), polymerase chain reaction, synergism.

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Роль респираторно-синцитиального вируса крупного рогатого скота в этиологии респираторных болезней на молочных комплексах

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РЕЗЮМЕ

Одним из этиологических агентов респираторных болезней является респираторно-синцитиальный вирус крупного рогатого скота (РСВ КРС). Возбудитель широко распространен во всех странах мира с интенсивным типом ведения животноводства и может вызывать патологию респираторного тракта самостоятельно или взаимодействуя с другими вирусами и бактериями. Актуальным является изучение распространения возбудителя на крупных молочных комплексах, выявление его во внутренних органах инфицированных животных, в том числе с количественной оценкой накопления в них. Целью работы было изучение особенностей распространения респираторно-синцитиальной инфекции, частоты выявления вируса в пробах биологического материала как в моноварианте, так и в ассоциациях с вирусами инфекционного ринотрахеита (ИРТ КРС) и вирусной диареи – болезни слизистых оболочек (ВД-БС КРС) крупного рогатого скота, бактериями семейства *Pasteurellaceae* на крупных молочных комплексах, неблагополучных по респираторным болезням животных, с определением концентрации вируса в органах респираторного тракта. В моноварианте РСВ КРС присутствовал в 9,2% исследованных проб биоматериала, в ассоциациях с вирусами ИРТ и ВД-БС КРС – в 1,4 и 5,2% проб соответственно. Количество проб, содержащих одновременно РСВ КРС и бактерии семейства *Pasteurellaceae*, составило 10,8%. Максимально вирус присутствовал в 26,6% проб от числа исследованных. Методом полимеразной цепной реакции в реальном времени геном вируса выявляли в легких (13,1%), в экссудате трахеи, бронхов и носовых синусов (6,0%), носовых выделениях (4,0%), бронхах (1,7%). Реже вирус присутствовал в пробах слизистой оболочки трахеи и бронхов (1,1%) и легочных лимфатических узлах (0,8%). Количественная оценка РНК РСВ КРС показала, что максимальное накопление вируса происходило в легких и носовых выделениях, что подтверждает данные об его тропизме к интерстицию легочной ткани.

Ключевые слова: Крупный рогатый скот, респираторные болезни, респираторно-синцитиальный вирус, полимеразная цепная реакция, синергизм.

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INTRODUCTION

Respiratory diseases of calves take one of the leading places among bovine pathologies in the Russian Federation. They cause death or a decrease in the growth rate of animals, result in additional expenditure on treatment, diagnostic and preventive measures [1, 2]. Bovine respiratory syncytial virus (BRSV) plays an important role in the structure of infectious respiratory diseases and this infection is widespread in all the countries with intensive animal husbandry [3, 4].

The causative agent of bovine respiratory syncytial virus infection (BRSV infection) is an enveloped RNA virus belonging to the *Pneumoviridae* family, the *Orthopneumovirus* genus, which mainly replicates in the cells of the respiratory epithelium.

Calves under 6 months of age are most susceptible to infection; however, adult animals can also get sick. The incubation period of BRSV infection is 2–5 days. At the beginning of the disease, animals may demonstrate the following symptoms: depression, fever, cough, rhinitis, rhinopharyngitis. In severe cases, a secondary infection can occur resulting in bronchitis, bronchiolitis, and pneumonia. The main complications of the infection may include emphysema, respiratory failure and acute fibrinous pneumonia. Post-mortem lesions are observed only in lungs.

Laboratory diagnosis of BRSV infection includes detection of the virus antigen in the pathological material from infected animals using immunofluorescence assay (IFA); or of the virus ribonucleic acid (RNA) using polymerase chain reaction (PCR), isolation of the virus in cell culture and determination of seroconversion to the virus in convalescent animals [5]. Due to great instability and weak ability of the pathogen to replicate in cell cultures, virological studies are not effective enough and require a lot of time and effort [6].

At present, it is crucial to study the BRSV infection epizootic situation in large dairy establishments (where imported cattle are held), as well as to study the tropism of the virus to respiratory tract with a quantitative assessment of its accumulation in them. The disease can occur independently or in association with other viral infections. Initial infection with BRSV in epithelial cells reduces the level of protection of the respiratory tract of animals and facilitates colonization and secondary infection of the lower respiratory tract by bacteria. The synergistic interaction between the virus and *Pasteurellaceae* bacteria is described [7, 8].

The aim of the research was to study BRSV infection spread, the frequency of the pathogen detection in samples of biological material alone and in associations with the viruses of infectious bovine rhinotracheitis (IBR) and bovine viral diarrhea/mucosal disease (BVDV), as well as with *Pasteurellaceae* bacteria in large dairy establishments affected by animal respiratory diseases; as well as to determine BRSV concentration in the respiratory tract.

MATERIALS AND METHODS

The studies were carried out in 2010–2020 in 7 large dairy establishments in Siberia, where specific prevention of BRSV infection was not carried out or only inactivated vaccines were used. The following samples of biological material were tested: nasal discharge, tracheal and bronchial exudates, pieces of bronchi and lungs, taken from dead animals or animal subject to emergency slaughter due to signs of respiratory failure. The samples were delivered frozen to the laboratory within 24 hours. Totally, 1,040 samples were tested; they were first ground in separate porcelain mortars with sterile sand, homogenized; then 10% suspensions were prepared on saline solution, centrifuged at 3,000 rpm for 15 minutes, then 100 µl of clarified supernatant was used to isolate RNA.

The virus RNA was isolated using the "RIBOsorb" kit (the Federal Budget Institution of Science of the Central Scientific Research Institute of Epidemiology of the Rospotrebnadzor) in accordance with the manufacturer's recommendations. Reverse transcription to obtain cDNA was performed using the "REVERTA-L" kit of the same manufacturer.

PCR with electrophoretic detection of results was used to detect genomes of three viruses in biomaterial samples, and real – time PCR was used to quantify BRSV RNA [6, 9]. The concentration of viral RNA in samples of biological material from sick animals was quantified relative to the mRNA level of the bovine GAPDH gene and expressed in $\log_{10}$ copies of viral RNA per 10^5 copies of GAPDH ($\log_{10}$ BRSV/GAPDH) [10].

Serological methods were used to study 6,000 sera samples. Antibodies to IBR and BVD viruses were detected using microneutralization assay in the continuous MDBK cell line according to the World Animal Health Organization (OIE, 2019) standard using "TK-A" strain and the cytopathogenic NADL strain as an antigen, and to BRSV – in the indirect hemagglutination test. For the purposes of serological screening, sera samples were taken from animals once, and to determine seroconversion they were taken twice with a 30-day interval. Persistent infection with BVDV was diagnosed when the viral RNA was detected in paired sera samples taken with a 30-day interval.

Pasteurellaceae bacteria were isolated in artificial culture media according to the guidelines for laboratory diagnosis of pasteurellosis in animals and birds, and their genotyping was performed using PCR [11].

RESULTS AND DISCUSSION

In order to determine how frequently BRSV is detected alone and in associations with IBR and BVD viruses and *Pasteurellaceae* bacteria (using PCR), we tested 1,040 biomaterial samples taken from dead and emergently slaughtered calves under 6 months of age with the signs of respiratory damage. In all animals post-mortem examination revealed acute fibrinous or catarrhal bronchopneumonia, and sometimes interstitial and pulmonary emphysema and signs of lung parenchyma destruction. The results of the study are presented in the Table.

The table shows that BRSV alone was present in 9.2% of the studied biomaterial samples, and in the associations with IBR and BVD viruses it was detected in 1.4 and 5.2% of the samples, respectively. The maximum number of samples (10.8%) contained both BRSV and *Pasteurellaceae* bacteria. In total, BRSV was detected in 26.6% of the tested samples [12, 13].

Respiratory syncytial virus alone was more often detected in lungs and bronchi, and in association with the IBR – in trachea and nasal mucosa, less often – in bovine lungs. Together with BVDV and *Pasteurellaceae* bacteria, BRSV was detected in lungs.

Table
Frequency of BRSV detection: alone and in association with IBR, BVDV and *Pasteurellaceae* bacteria using PCR with electrophoretic detection [12, 13]

Таблица
Частота выявления РСВ КРС в моноварианте и в ассоциациях с вирусами ИРТ, ВД-БС КРС и бактериями семейства *Pasteurellaceae* при помощи ПЦР с электрофоретической детекцией [12, 13]

n = 1,040

Pathogen, association of pathogens	Number of positive samples	Percent of positive samples from the number of samples tested
BRSV alone	96	9.2
BRSV + IBR	15	1.4
BRSV + BVDV	54	5.2
BRSV + *Pasteurellaceae* Including bacteria: *Pasteurella multocida* *Mannheimia haemolytica*	112 42 70	10.8 4.0 6.7
Total	277	26.6

Many authors assign a special role in the synergism of infectious agents to BVDV, which, due to its immunosuppressive effect, can increase the susceptibility of animals to infection with other viral and bacterial pathogens [7, 8, 14, 15].

According to our data, the level of infection of animals with BVDV is 90% and above in large dairy establishments, where about 3% of calves are persistently infected. The percentage of animals (of all sex and age groups) seropositive to BRSV in such herds is 67.5% on average, and the virus genome is detected more often than in herds without persistently infected animals [9].

Thus, large dairy establishments demonstrate a mixed presence of BVDV and BRSV. A correlation was revealed between the level of BVDV in animals, i.e. between the presence of animals persistently infected with this pathogen in the examined establishments, and the frequency of clinical signs of respiratory diseases in calves that occur due to BRSV [12].

Often, after a predisposing viral infection, secondary bacterial bronchopneumonia develops, since damage to the epithelium of the respiratory tract leads to a violation of mucociliary clearance and facilitates the movement of bacteria to the lower respiratory tract. In addition, respiratory viruses suppress the phagocytic activity of alveolar macrophages and disrupt intracellular bactericidal processes. Also, respiratory viruses can promote bacterial adhesion, enhance the expression of surface proteins of host cells, which can then be joined by bacteria [7, 16].

Basically, secondary bacterial bronchopneumonia develops due to the presence of *Pasteurellaceae* bacteria, namely *Mannheimia haemolytica* and *Pasteurella multocida* [8, 9, 15].

An important aspect of the BRSV infection pathogenesis is that it suppresses non-specific mechanisms of the respiratory immune defense, and it initiates and enhances bacterial colonization of the lungs after its primary replication. The virus can independently cause bronchitis, pneumonia and emphysema, but its main feature is immunosuppression and an ability to provide preconditions for bacterial pneumonia, in particular, pulmonary pasteurellosis [5, 8].

The results of the conducted research show that BRSV infection is common in large dairy establishments in Siberia and can occur both alone and in various associations [17]. The synergistic interaction of microorganisms of different classes plays a significant role in the occurrence of mass bronchopneumonia in cattle under natural conditions. When developing effective measures of specific prophylaxis for this bovine disease, especially when importing animals from different sources, it is important to understand and decipher the mechanisms that contribute to the development of mixed forms of infections.

Previously, we studied BRSV distribution in the respiratory tract of calves with respiratory syncytial infection using PCR with electrophoretic detection of amplification products, but the concentration of the virus RNA could not be determined due to the limitations of the method [3].

To study the virus distribution in the upper and lower respiratory tract, positive samples of biomaterial were additionally tested in real-time PCR and it was found that the BRSV genome was more often present in lungs (13.1% of the number of tested samples). In addition, the virus was detected in the exudate from trachea, bronchi and nasal sinuses, which accounted for 6.0% of the samples. The percentage of virus detection in nasal discharge was 4.04%, and in the bronchi – 1.7%. The virus was less often detected on the tracheal and bronchial mucosa (1.1%) and in pulmonary lymph nodes (0.8%). The data obtained demonstrate a wide distribution of the virus in the organs of the upper and lower respiratory tract.

Virus quantification in different parts of the respiratory tract of infected animals was of great interest. The maximum concentrations of the virus genome were detected in lungs ($1.3 \pm 0.5 - 4.8 \pm 0.47$ $\log_{10}$ copies of BRSV RNA/GAPDH), nasal discharge ($1.5 \pm 0.75 - 2.1 \pm 0.25$ $\log_{10}$ copies of BRSV RNA/GAPDH) and exudates from trachea, bronchi, and nasal sinuses ($0.3 \pm 0.21 - 2.8 \pm 0.15$ $\log_{10}$ copies of BRSV RNA/GAPDH). Different virus concentrations detected during the research in the biomaterial samples may indicate that animals were sampled at different stages of the infectious process.

The above facts show how important respiratory syncytial virus is among bovine respiratory diseases and demonstrate its role in the pathogenesis of mixed respiratory diseases.

CONCLUSION

The results of the conducted research improve understanding of respiratory syncytial infection in animals in large dairy establishments and of its role in the pathogenesis of mono- and mixed infections of the bovine respiratory tract. BRSV was detected in 26.6% of biomaterial samples from sick and dead calves under 6 months of age; samples were taken during mass outbreaks of respiratory diseases, in particular during acute fibrinous bronchopneumonia. The virus alone was detected in 9.2% of cases, and in associations with IBR and BVDV in 1.4 and 5.2% of biomaterial samples, respectively. The number of samples containing BRSV and *Pasteurellaceae* bacteria was 10.8%, which confirms the synergistic interaction between infectious agents of different nosological groups.

In some establishments, the frequency of BRSV infection directly depended on the level of animal infection with BVDV, as well as on the presence of animals persistently infected with this virus in herds [12].

Quantitative analysis of BRSV RNA in the tested biomaterial samples showed its maximum accumulation in lungs and nasal discharge, and it confirms the data on the virus tropism to the pulmonary interstitium, and this contributes to the occurrence of acute fibrinous bronchopneumonia. Quantification of viruses and bacteria with the help of real-time PCR can be a useful tool to study pathogenesis of mixed viral-bacterial infections in the wild.

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Effectiveness of vaccines produced by the Federal State-Financed Institution "ARRIAH" against topical genotype VII Newcastle disease viruses

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SUMMARY
In 2019, the situation regarding Newcastle disease in the Russian Federation worsened radically due to the spread of NDV subgenotype VII-L throughout the country from the Primorsky Krai to the Kursk Oblast. As a result, 17 infected settlements with backyard farms where unvaccinated poultry was kept were registered. In this study, immunogenicity of the vaccines produced by the FGBI "ARRIAH", as well as the effectiveness of various vaccination schedules to prevent genotype VII NDVs, relevant for the Russian Federation, was studied. It is known that the currently circulating ND agent is significantly more virulent compared to the viruses isolated in previous years, and it is able to bypass the immunity provided by live vaccines. Test results demonstrated that the vaccines against genotype VII NDVs produced by the FGBI "ARRIAH" are highly immunogenic, which allows to effectively prevent the disease when using them as part of a standard vaccination schedule. A 2-dose vaccination schedule using live vaccine from the La Sota strain as well as the "complete" vaccination schedule using inactivated vaccines provides immunity in 100% of chicks. The use of live vaccines in a single- and double-dose vaccination schedules prevents mortality and clinical disease in poultry, but does not prevent virus replication, while the addition of an inactivated vaccine to the immunization schedule does prevent the replication of the virulent virus. Thus, the use of domestically produced live and inactivated vaccines, primarily the ones containing the La Sota strain, with the following control of the immunity level and booster vaccination, if required, is the main tool for the disease control.

Keywords: Virulent genotype VII Newcastle disease virus, ND vaccines, effectiveness of ND vaccines.

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Эффективность вакцин против ньюкаслской болезни производства ФГБУ «ВНИИЗЖ» в отношении актуальных вирусов VII генотипа

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РЕЗЮМЕ

В Российской Федерации в 2019 году произошло резкое обострение ситуации по ньюкаслской болезни птиц с распространением вируса субгенотипа VII-L по всей территории страны – от Приморского края до Курской области. В итоге зарегистрировано 17 неблагополучных пунктов, где содержалось невакцинированное поголовье в личных подсобных хозяйствах граждан. В данной работе оценивали иммуногенность вакцин производства ФГБУ «ВНИИЗЖ», а также эффективность различных схем вакцинации для профилактики ньюкаслской болезни в отношении актуальных для Российской Федерации вирусов VII генотипа. Известно, что вирулентность циркулирующего в настоящее время возбудителя ньюкаслской болезни заметно возросла по сравнению с вирусами, выделенными в предыдущие годы, и он способен преодолевать поствакцинальный иммунитет, создаваемый живыми вакцинами. В результате исследований было установлено, что вакцины производства ФГБУ «ВНИИЗЖ» обладают высокой иммуногенной активностью в отношении вирусов VII генотипа, что позволяет эффективно профилактировать эту болезнь при их использовании в составе стандартных схем вакцинации. Схема вакцинации с двукратным применением живой вакцины из штамма «Ла-Сота» формирует иммунитет у 100% цыплят, так же как и «полная» схема вакцинации с использованием инактивированных вакцин. Применение живых вакцин в схеме вакцинации с однократным и двукратным введением предотвращает гибель птиц и клиническое проявление болезни, однако не препятствует репликации вируса, в то время как добавление в схему иммунизации инактивированной вакцины предотвращает, кроме того, и репликацию вирулентного вируса. Таким образом, использование живых и инактивированных вакцин отечественного производства, прежде всего на основе штамма «Ла-Сота», с последующим контролем напряженности иммунитета и проведением ревакцинаций по показаниям является главным инструментом в борьбе с заболеванием.

Ключевые слова: Вирулентный вирус ньюкаслской болезни VII генотипа, вакцины против ньюкаслской болезни, эффективность вакцин против ньюкаслской болезни.

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INTRODUCTION

Newcastle disease (ND) is a highly contagious viral disease that affects birds, including domestic poultry (mainly chickens). The virus affects the digestive, respiratory, and nervous systems. The disease is registered throughout the world, it is included in the OIE list of notifiable diseases, as it causes huge economic losses and hinders international trade [1–3].

To date, NDV strains have been classified into classes: Class 1 – avirulent strains (9 genotypes) and Class 2 – vaccine and virulent strains (at least 10 genotypes). This is the most diverse and constantly evolving group of viruses. Virulent viruses of the genus *Avian orthoavulavirus* 1 (AOAV1), formerly known as *Avian avulavirus* 1, *Avian paramyxovirus* 1, or Newcastle disease viruses, can infect and cause disease in a wide range of domestic and wild avian species worldwide. *Avian orthoavulavirus* 1 has high genetic variability. Currently, the AOAV-1 is divided into 2 classes containing more than 20 genotypes, some of which are further divided into about 30 subtypes. In recent decades, the most relevant for poultry farming have been genotype VII, widely distributed in the countries of the Old World, and genotype V (including the newly isolated genotype XIX), circulating in the Americas.

The emergence of new virulent class 2 genotype strains that cause epidemics can be explained by the fact that viruses of different genotypes develop simultaneously in different geographical areas around the world, which is facilitated by the large diversity of avian species susceptible to NDV [1, 4]. Over the past 20 years, NDV epidemics in many Asian and European countries have been caused by different subtypes of genotype VII viruses [1–3]. Two lineages of class 2 genotype VII (previously called subgenotypes VIIh and VIIi) were identified in Indonesia in 2010 [5]. Later, one of the lineages was introduced into Malaysia [6], China [7], a number of countries in South Africa [8] and Russia (NDV/chicken/Kaliningrad/184/2013). The other subgenotype was identified in Pakistan [9], India [10], Israel [11], Libya [12], Turkey, Georgia and Bulgaria [13].

In the Russian Federation, this genotype VII NDV caused an outbreak on a poultry farm in the Amur Oblast in 2006 for the first time. Currently it is used as a challenge strain [14]. In the following years, genotype VII NDV caused sporadic outbreaks in poultry kept on backyard farms in different regions of the country. ND outbreaks caused by subgenotype VIIi viruses were first reported in the Republic of Crimea at the end of 2015 and continued throughout 2016, which at that time was the largest and longest NDV epidemics in poultry in Russia over the past few decades (21 infected settlements). This suggests panzootic potential of both virus groups [15]. It is worth mentioning that viruses with genomes similar to those of the isolates belonging to these groups were detected later than in the mid-90s in the countries of the Far East and Southeast Asia, or sporadically on the islands of Indonesia. This suggests the existence of a permanent virus reservoir in the tropical area of Southeast Asia, in which new forms of virulent AOAV-1 develop. From time to time, they leave the reservoir.

In 2019, the situation regarding Newcastle disease in the Russian Federation worsened radically due to the spread of NDV subgenotype VII-L (VII 1.1 according to the

new classification [16]) throughout the country from the Primorsky Krai to the Kursk Oblast. As a result, 17 infected settlements with backyard farms where unvaccinated poultry was kept were registered.

NDV isolates of subgenotype VII-L are most closely related to some isolates from Iran, designated cluster VII-L [17]. Iranian scientists have shown high similarity between the isolates, the fact that they belong to genotype VII, their compliance with the criteria for new subgenotype identification, as well as close phylogenetic relationship with the already known and widespread subgenotype VIId NDVs. Later, the same authors [17] significantly increased the number of the studied isolates belonging to this group, showed their distribution in almost all the Iranian provinces and the phylogenetic analyses of partial F gene sequences revealed that the isolate recovered in the country back in 1999 was evolutionarily similar to the isolates belonging to this group. F. Sabouri et al. [18], as well as A. Molouki et al. [19], demonstrated that subtype VII-L viruses were common both on small-scale and commercial poultry farms. The emergence of the new genotype VII subline in this region speaks of the fact that similar processes may occur in other places, since subgenotype VIId viruses are endemic in many countries of Eurasia and Africa, and have been also found in South America [20].

There have been no registered cases of NDV of this subgenotype in industrial poultry farming in Russia so far.

In most countries, including the Russian Federation, vaccination of commercial poultry against NDV is mandatory [1, 4, 21]. The terms of the primary vaccination are determined based on the level of maternal antibodies. As a rule, blood sera are tested by the hemagglutination inhibition test (HI) 14–21 days after the primary vaccination. Immunization is considered successful if at least 80% of vaccinated poultry has an antibody titer of at least 3 $\log_2$ [22]. When using inactivated vaccines, the antibody titer should be at least 5 $\log_2$ [23].

Insufficient and heterogeneous specific antibody levels following routine vaccination may be due to the use of an inadequate vaccine strain in a specific epidemic situation, incomplete dose administration, technical failures, vaccination of poultry with high maternal antibody levels, and other reasons.

The aggravation of the epidemic situation in 2019 and the spread of genotype VII NDV throughout the Russian Federation served the basis for the experimental infection of poultry with actual viruses of the mentioned genotype to assess the protectivity of live and inactivated La Sota vaccines produced by the FGBI "ARRIAH" (Vladimir, Russia) following different vaccination schedules.

MATERIALS AND METHODS

Vaccines. The following ND vaccines (live and inactivated) produced by the FGBI "ARRIAH" were used in the study:

– live dry vaccine against Newcastle disease from La Sota strain, batch No. 140520 (release date 05.2020);

– combined inactivated emulsion vaccine against Newcastle disease, avian infectious bronchitis and egg drop syndrome-76, batch No. 010320 (release date 03.2020).

Viruses. Three genotype VII NDVs relevant to the Russian Federation were used in the challenge: NDV/chicken/Rus/Crimea/54/17, NDV/chicken/Rus/Krasnodar/9/19, NDV/chicken/Rus/Kaliningrad/184/13, which were further assigned the following names: "Crimea", "Krasnodar" and "Kaliningrad", respectively.

Experimental animals. Egg-producing chicks aged 14–21 days without antibodies to NDV, obtained from a poultry farm free from infectious avian diseases were used in the experiment.

All tests in animals were carried out in strict compliance with the interstate standard for keeping and care of laboratory animals GOST 33215-2014, 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.

Experiment design. Four experimental groups of chicks were formed by random sampling. Group No. 1 (30 chicks) were vaccinated with a single dose of the La Sota vaccine. Group No. 2 (30 chicks) were vaccinated with two doses of the La Sota vaccine, booster immunization being carried out 28 days after the primary vaccination. Group No. 3 (30 chicks) were vaccinated with two doses of live vaccine against ND and then 21 days later – with a single dose of inactivated vaccine against ND. Group No. 4 (90 chicks) served as a negative control and consisted of unvaccinated chicks.

The following abbreviations were used to identify the vaccination schedules: 1 LV – single-dose immunization with live vaccine; 2 LV – 2-dose immunization with live vaccine; 2 LV+1 IV – 2-dose immunization with live vaccine and single-dose immunization with inactivated vaccine.

Live vaccines were administered intranasally at a dose of 6.7 lg EID_{50}, which corresponds to one immunizing dose of the La Sota vaccine produced by the FGBI "ARRIAH"; inactivated vaccine (single dose, 0.5 ml) was administered intramuscularly.

Control of the vaccine immunogenicity. The vaccine immunogenicity was assessed based on the challenge and serological test results.

The chicks were challenged in accordance with the OIE Manual for Diagnostic Tests and Vaccines for Terrestrial Animals [24]. Chicks from different experimental groups were challenged with virulent NDVs of genotype VII. The virulent strains were used at the following infective doses: NDV/chicken/Rus/Kaliningrad/184/13 – 5.9 lg EID_{50}; NDV/chicken/Rus/Crimea/54/17 – 6.6 lg EID_{50}; NDV/chicken/Rus/Krasnodar/9/19 – 6.9 lg EID_{50}. Chicks were inoculated intramuscularly with 0.5 ml of the inoculum and were monitored for 7–8 days. Each time, 60 chicks were randomly selected to make 6 experimental groups (10 chicks per group) subjected to the challenge:

group No. 1 – vaccinated chicks challenged with NDV/chicken/Rus/Crimea/54/17;

group No. 2 – vaccinated chicks challenged with NDV virus/chicken/Rus/Krasnodar/9/19;

group No. 3 – vaccinated chicks challenged with NDV virus/chicken/Rus/Kaliningrad/184/13;

group No. 4 – unvaccinated chicks challenged with NDV/chicken/Rus/Crimea/54/17 virus;

group No. 5 – unvaccinated chicks challenged with NDV virus/chicken/Rus/Krasnodar/9/19;

group No. 6 – unvaccinated chicks challenged with NDV/chicken/Rus/Kaliningrad/184/13.

Challenge was performed 21–28 days after each vaccination.

For serological studies, chick serum samples were collected from different experimental groups, tested by HI test using an HI kit for the detection of antibodies to Newcastle disease virus produced by the FGBI "ARRIAH".

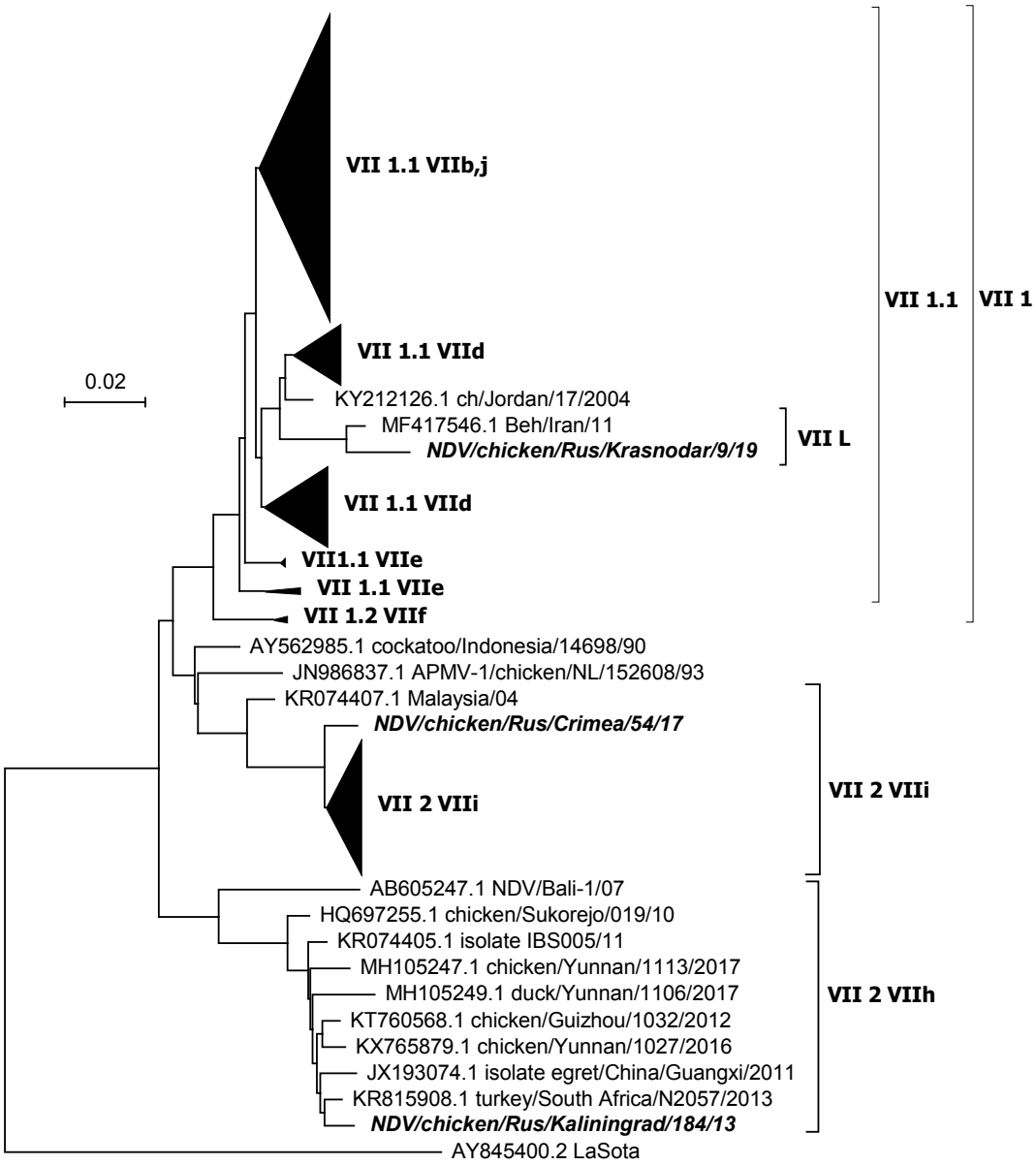

Fig. 1. Phylogenetic position of genotype VII isolates that have caused ND outbreaks in Russia in recent years. The dendrogram for complete F gene ORF sequences of 3 Russian and 304 previously published strains and isolates was obtained using the NJ program, MEGA 6.0 package. Russian isolates are shown in bold and italics. On the right are the names of phylogenetic groups according to D. G. Diel et al. [25] *(Roman numerals and Latin letters) and K. M. Dimitrov et al.* [16] *(Roman and Arabic numerals). Phylogenetic groups that do not contain the studied isolates are shown in a contracted form for convenience*

Рис. 1. Филогенетическое положение изолятов генотипа VII, вызвавших в России вспышки НБ в последние годы. Дендрограмма получена для полных последовательностей ОРС гена F 3 российских и 304 ранее опубликованных штаммов и изолятов с помощью программы NJ пакета MEGA 6.0. Российские изоляты выделены жирным шрифтом и курсивом. Справа приведены названия филогенетических групп по D. G. Diel et al. [25] *(римские цифры и латинские буквы) и по K. M. Dimitrov et al.* [16] *(римские и арабские цифры). Филогенетические группы, не содержащие изучаемых изолятов, для удобства показаны в свернутом виде*

Serum samples were collected before vaccination and before each challenge test, as well as 7–8 days after the challenge test from the survived chicks.

Nucleotide sequences of virulent NDV genes used for the challenge. Figure 1 shows the nucleotide sequences of NDV isolates and strains, characterized at the FGBI "ARRIAH" or published in the GenBank databases of the NCBI (www.ncbi.nlm.nih.gov/nucleotide/) and GISAID EpiFlu (https://www.gisaid.org/).

Nucleotide and their corresponding amino acid sequences were analyzed using BioEdit, version 7.0.5.3. Sequence alignment was performed using the ClustalW multiple alignment program. The phylogenetic tree was constructed using the NJ algorithm, MEGA package, version 6.0.

Analysis of test results. Immunogenicity of the vaccines and the effectiveness of the vaccination schedules used were assessed by measuring vaccination effectiveness, as well as based on the serological test results.

Table 1
Results of the challenge in chickens immunized following different vaccination schedules

Таблица 1
Результаты контрольного заражения цыплят, иммунизированных с применением различных схем вакцинации

Vaccination schedule	NDV virulent strain			Vaccination effectiveness, % $M \pm m$ ($n = 3$)
	"Crimea"	"Krasnodar"	"Kaliningrad"	
1 LV	2/10*	6/10	0/10	73 ± 18
2 LV	0/10	0/10	0/10	100
2 LV+1 IV	0/10	0/10	0/10	100
control	10/10 ($n = 3$)	10/10 ($n = 3$)	10/10 ($n = 3$)	0

* The ratio of the dead to the total number of infected chickens (Отношение павших к общему количеству зараженных цыплят).

Vaccination effectiveness (E) was calculated using the equation:

$$E = (n - n_i)/n \times 100\%,$$

where n_i is the number of dead chicks in the group, and n is the total number of chicks in the group.

To characterize the pathogenic effect of the virulent ND viruses, they used lethality rate calculated for a group of chicks vaccinated with a single dose of live vaccine. Lethality rate is the ratio between the number of dead animals and the number of susceptible animals, expressed as a percentage.

Lethality rate (L) was calculated using the equation:

$$L = n_i/n \times 100\%,$$

where n_i is the number of dead chicks in the group, and n is the total number of chicks in the group.

During statistical processing, the average vaccination effectiveness, lethality, antibody titers, and standard errors of the mean were calculated and analyzed using the Student's t-criterion to achieve 95% confidence level.

RESULTS

Immunogenicity of the vaccines and effectiveness of the vaccination schedules used in the experiment was assessed by the resistance to the challenge with virulent NDVs, as well as by serological test results.

Table 1 presents summarized results on the resistance of vaccinated chicks to the challenge with virulent NDVs. According to the table the effectiveness of single-dose vaccination in the groups varied from 40 to 100% and was 73% on average. The greatest effectiveness of single-dose vaccination was demonstrated for the Kaliningrad strain (10 out of 10 chicks survived), and the lowest – for the Krasnodar strain (4 out of 10 chicks survived).

Also, from the data shown in Table 1, it follows that vaccination effectiveness varied from 73 to 100%, depending on the schedule used. The lowest effectiveness was observed after a single dose of the live vaccine and the highest – after the administration of two doses of the live vaccine and an additional dose of the inactivated vaccine.

At the same time, in all the unvaccinated control groups, in three-stage testing, 100% lethality in chicks was observed on day 3–7 post-challenge.

Figure 2 presents lethality rate in chicks in group 1 LV after their challenge with genotype VII NDVs. According to the diagram, as well as based on the regression equation and the determination coefficient $R = 0.84$ or 84%, a high degree of correlation between lethality and the infective dose was established.

Figure 3 shows the correlation between the distribution of NDV antibody titers in experimental groups and the vaccination schedule used. The diagram demonstrates that the vaccination schedule 2 LV+1 IV resulted in high antibody titers, with the mean titer value of 11.9 $\log_2$. Following other schedules, when the chicks had received one or two live vaccine doses, the mean antibody titer values were 5.2 and 4.8 $\log_2$, respectively.

Table 2 presents results of serological tests to determine anti-NDV antibody titres in poultry sera by HI test after following different vaccination schedules, as well as after the challenge test.

The data in Table 2 demonstrates that the antibody titers after single (5.2 ± 0.3 $\log_2$) and double (4.8 ± 0.2 $\log_2$) vaccination were almost the same, since there was no statistical difference ($P > 0.05$). Vaccination of chicks with the inactivated vaccine resulted in a significant (statistically significant) increase in antibody titers ($P < 0.001$).

After the challenge, the antibody titers in group 1 LV and 2 LV increased significantly and exceeded the initial values by 48 and 119 times, respectively; as for the group 2 LV+1 IV the antibody titers were comparable to those before the challenge – 11.9 $\log_2$.

DISCUSSION OF THE RESULTS

Single-dose vaccination with the live vaccine produced by the FGBI "ARRIAH" resulted in high titers of humoral antibodies, which were comparable to those developed after two-dose vaccination. However, the effectiveness of single-dose vaccination was the lowest (73%), whereas after the administration of two doses of live vaccine and an additional dose of the inactivated vaccine, the effectiveness against virulent genotype VII NDVs was the greatest and amounted to 100%.

It should be noted that genotype VII NDVs' pathogenicity depended on the infective dose, and this could be observed in chicks vaccinated with a single dose of the

live vaccine. Further in the studies, when characterizing the effectiveness of the 2 LV and 2 LV+1 IV vaccination schedules, this correlation was mitigated. It should also be noted that it is almost impossible to find such virus concentration (about 5.9 lg EID_{50} or ~1,000,000 viral particles) in the wild, so it is most likely that single-dose vaccination with live La Sota vaccine will protect poultry from the infection with virulent genotype VII NDVs.

Apparently, two-dose immunization with live vaccine and the additional administration of the inactivated vaccine results in higher immunity levels and specific immunity, characterized mainly by "late" immunoglobulin G, which is more effective against virulent NDVs. However, immunization of poultry with live vaccines did not completely prevent the virulent virus replication, as evidenced by the 50–100 times increase in humoral antibody titers 7–8 days post-challenge.

The so-called "complete" vaccination schedule, using live and inactivated vaccines, resulted in high humoral antibody titers, prevented the virulent virus replication, and provided 100% protection against the infection.

CONCLUSION

It was found that vaccines are effective for the prevention of Newcastle disease, caused by virulent genotype VII viruses, when used following a specific vaccination schedule. Thus, a two-dose vaccination schedule using live vaccines, prevented poultry death and clinical manifestation of the disease, but did not prevent virus replication. While the vaccination schedule using live and inactivated vaccines prevented death, the development of clinical disease signs and replication of the virulent virus.

Therefore, vaccines against ND produced by the FGBI "ARRIAH" are highly immunogenic and effective in the prevention of disease caused by genotype VII NDVs.

The data obtained indicate that the currently circulating NDV is significantly more virulent than those isolated in previous years, and it is able to bypass the post-vaccination immunity induced by live vaccines. Therefore, the use of adequate domestically produced live and inactivated vaccines, primarily those containing the La Sota strain, with the following control of the immunity level and booster vaccination, if required, is the main tool for the disease control.

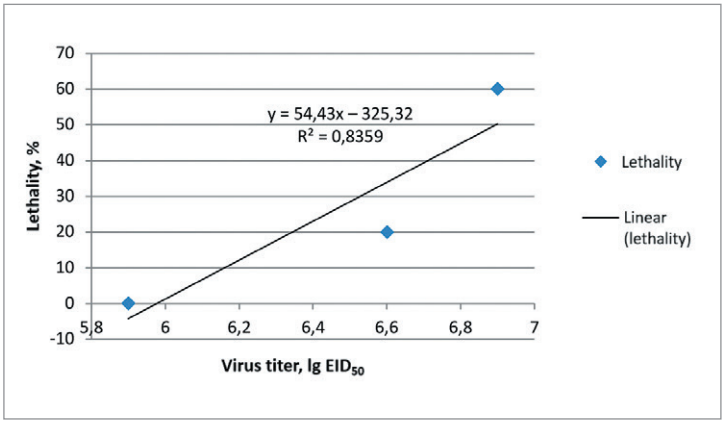

Fig. 2. Relationship between the infectious dose and the mortality rate in single-dose vaccinated chickens

Рис. 2. Влияние заражающей дозы на показатель летальности однократно вакцинированных цыплят

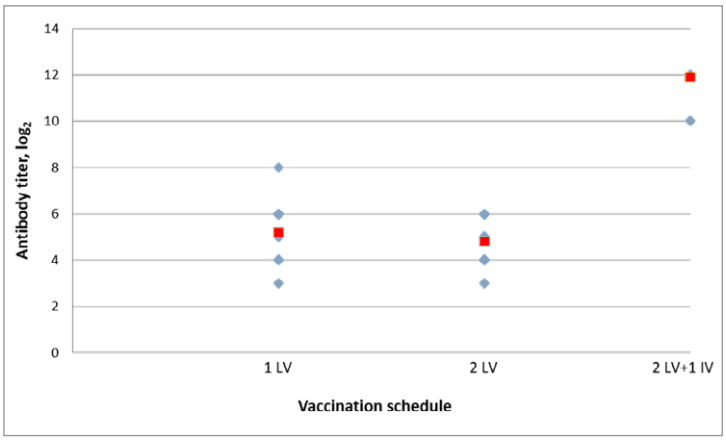

Fig. 3. Distribution of antibody titers by groups, according to the vaccination schedules used. The red markers indicate the average values of the corresponding set of variate values

Рис. 3. Распределение титров антител по группам, соответственно примененным схемам вакцинации. Красными маркерами выделены средние значения соответствующих вариационных рядов

Table 2
Serological response in chickens to vaccination following different ND vaccination schedules

Таблица 2
Серологический ответ цыплят на иммунизацию с использованием различных схем вакцинации против НБ

Vaccination schedule	Antibody titer (log_2) at different sample collection time points		
	0 b/v	21–28 p/v	7–8 p/ch
1 LV	3.2 ± 0.6	5.2 ± 0.3	10.8 ± 0.3
2 LV		4.8 ± 0.2	11.7 ± 0.1
2 LV+1 IV		11.9 ± 0.1	11.9 ± 0.1

b/v – days before vaccination (дней до вакцинации), p/v – days post-vaccination (дней после вакцинации), p/ch – days post-challenge (дней после контрольного заражения).

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Epidemic situation of cestodiasis in domestic reindeer on reindeer farms in the Murmansk Oblast

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SUMMARY

The paper presents the results of the cestodiasis epidemic situation in domestic reindeer in the farms of the Murmansk Oblast. The studies were performed in 2018–2019 during the routine slaughter of reindeer at slaughter houses APC "Tundra" and APC HFE SEN "Olenevod" located in settlements Lovozero, Krasnoschelye, and Sosnovka. Totally 4,048 carcasses of domestic reindeer were tested, 2,812 out of them – in Lovozero, 396 – in Sosnovka, and 840 – in Krasnoschelye. During the meat inspection the parenchymal organs were examined for cestode cysts. When detected they were sampled and gross specimens were prepared using standard parasitological methods. 56 samples of internal organs of deer suspected in tapeworm infestation were collected from the inspected carcasses, in 25 of them tapeworms were detected and in the rest of the samples parasites were not detected. The tapeworm species were determined at the Department of Veterinary Biosiences, Faculty of Veterinary Medicine, University of Helsinki. The test performed revealed echinococcosis (*Echinococcus canadensis*) and cysticercosis (*Taenia hydatigena*). Most lesions were detected in liver where the agent's larvae cysts are observed. It was established that the level of domestic reindeer infestation with the agents of cysticercosis in APC "Tundra" was 0.5%, echinococcosis – 0.04%, in APC HFE SEN "Olenevod" cysticercosis was diagnosed in 0.81% cases, echinococcosis was not detected. On the whole 0.62% of reindeer on reindeer farms were infested with cestodes. Measures taken for prevention of helminth infestation in domestic and farm animals bear good results.

Keywords: Epidemic situation, cestodiasis, helminth infestation, reindeer, *Taenia hydatigena*, *Echinococcus canadensis*.

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Эпизоотическая ситуация по цестодовой инвазии домашних северных оленей в оленеводческих хозяйствах Мурманской области

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РЕЗЮМЕ

В статье представлены результаты анализа эпизоотической ситуации по цестодозам домашних северных оленей в хозяйствах Мурманской области. Исследования проводили в период с 2018 по 2019 г. во время планового убоя северных оленей на убойных пунктах СХПК «Тундра» и СХПК ОПХ МНС «Оленевод», расположенных в поселениях Ловозеро, Краснощелье, Сосновка. Всего было обследовано 4048 туш домашних северных оленей, из них 2812 – в Ловозеро, 396 – в Сосновке, 840 – в Краснощелье. При ветеринарно-санитарной экспертизе проводили внешний осмотр паренхиматозных органов на предмет наличия на них цестодовых пузырей. При их обнаружении производили отбор проб и готовили макропрепараты по общепринятым в паразитологии методикам. Из числа обследованных туш было отобрано 56 проб внутренних органов оленей с подозрением на наличие цестод, в 25 из них обнаружены гельминты, в остальных образцах паразиты не выделены. Видовую принадлежность гельминтов определяли на кафедре ветеринарно-биологических наук факультета ветеринарной медицины Хельсинкского университета. В результате проведенных исследований из гельминтозных заболеваний были выявлены эхинококкоз (*Echinococcus canadensis*) и цистицеркоз (*Taenia hydatigena*). Преимущественно была поражена печень, где в пузырчатой форме паразитируют личинки возбудителя. Установлено, что инвазированность домашних северных оленей возбудителями цистицеркоза в СХПК «Тундра» составила 0,5%, эхинококкоза – 0,04%, в СХПК ОПХ МНС «Оленевод» цистицеркоз диагностировали в 0,81% случаев, эхинококки не выделены. В целом по оленеводческим хозяйствам цестодами поражено 0,62% оленей. Мероприятия по предупреждению распространения гельминтозов среди домашних и сельскохозяйственных животных проводятся достаточно эффективно.

Ключевые слова: Эпизоотическая ситуация, цестодоз, гельминтоз, северный олень, *Taenia hydatigena*, *Echinococcus canadensis*.

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INTRODUCTION

Necrobacteriosis, oedemagenosis, cephenomyosis and helminthiasis should be singled out among the most frequently registered diseases in reindeer herds, which cause significant economic damage to reindeer husbandry. They reduce the animal performance and often cause their death [1–3].

Helminthiases occupy a special place among the diseases of domestic reindeer. They reduce weight gain during feeding of animals, often cause their emaciation, and also worsen the quality of meat and leather, and fur products [4]. Deer infected with helminths at 5 months of age weigh 6.2 kg less [5] than the healthy ones, and adults – 16.4 kg less [6], the difference in meat yield is on average 3.8 and 10.9 kg respectively. Due to helminthiasis during the autumn slaughter of deer, 0.3–2% of carcasses, 25–40% of liver, 10–20% of hearts and lungs are discarded, which in some northern regions amounts to direct economic damage, measured in hundreds of tons. Worm infestations not only reduce the productivity of deer, but also increase their susceptibility to other diseases, such as necrobacteriosis, bronchopneumonia [7, 8]. It is known that 74 species of helminths can infect reindeer, 35 of which are found only in this animal and are specific for it [9].

Echinococcosis and cysticercosis are widespread among helminthic diseases in reindeer husbandry. In this case, mainly the liver and lungs are affected, where the pathogen larvae cysts are observed. In the tape stage, helminths parasitize in the intestines of carnivores (dogs, wolves, polar foxes) and humans. At this stage, parasites persist for up to 18 months in the external environment and enter the deer's body with food. Effective measures to combat the worm cysts have not been developed. Affected parenchymal organs and tissues are discarded and destroyed. Control measures are aimed at de-worming treatments of dogs and destruction of faeces [10].

Compliance with modern requirements of veterinary and sanitary rules when working in the livestock industry has significantly improved the epidemiological situation in the country. However, there are regions with a high incidence of cysticercosis and echinococcosis in animals.

In Yakutia, the *Taenia parenchimatosa* infection rate of reindeer reaches 84.6%, and *Echinococcus granulosus* – up to 17.2% [3, 10]. According to the V. A. Bolshakov and I. I. Grigoriev's observations [5, 7], in the mountain-taiga zone of Yakutia cysticercosis infects 23.6–84.6% of young animals at the age of 6–8 months and 7.3–46.7% of adult deer, and echinococcosis – up to 7.5% of individual animals.

In Western Taimyr, the incidence of cysticercosis in reindeer is 61.5% [11].

In the Kabardino-Balkarian Republic, the incidence of echinococcosis in wild ungulates (chamois, deer, roe deer) was registered at the level of 18.5–50%, and cysticercosis – 7.4–11.8% [12].

In the conditions of the Central Chernozem Region (Voronezh Reserve) in the 70s–80s of the last century, the prevalence of echinococcosis in wild ungulates was less than 1%, but by 2017 it increased to 10% among elks. This has been associated with an increase in echinococcosis

infections in stray dogs. The average long-term infection with cysticercosis (*Taenia hydatigena*) was 19.9–86.2% in various ungulates (deer, roe deer, elk) [13, 14].

Thus, in some regions of the country, foci of the pathogens of helminthiases of farm animals are maintained. At the same time, the natural chain of disease transmission between the definitive (wolf, dog) and intermediate (deer, elk, roe deer) hosts is preserved.

Timely detection of echinococcosis and cysticercosis infested farm animals and their protection from pathogens are directly related to human health. So, in 2019, 451 cases of echinococcosis (0.31 per 100 thousand population) and 65 cases of alveococcosis (0.04 per 100 thousand population) infestations were detected in humans in the Russian Federation. Teniarinhoses (causative agent – *Taenia saginata*) and teniasis (causative agent – *Taenia solium*) account for up to 0.31% of all cases of helminthiasis in humans [15].

Analysis of the long-term incidence of echinococcosis in the constituent entities of the Russian Federation showed that the incidence of this helminthiasis exceeds the national average in Yamalo-Nenets (7.3 times), Chukotka (4.8 times), Khanty-Mansiysk (1.6 times) autonomous okrugs, Stavropol (1.8 times), Perm (1.1 times), Altai (1.6 times) territories, Kabardino-Balkarian (2.0 times), Karachay-Cherkess Republics (9.7 times), Republics of Bashkortostan (3.6 times), Altai (3.5 times), Sakha (Yakutia) (2.8 times), Kalmykia (2.5 times), Dagestan (1.8 times), Orenburg (6.9 times), Saratov (4.5 times), Astrakhan (4.1 times), Kurgan (2.4 times), and Kirov (1.1 times) Oblasts [16].

The main reasons for the disease spread remain violations of the rules for farm animal slaughter, the ineffectiveness of de-worming treatment of dogs and disinfection of natural environments. The main victims are workers who are in direct contact with farm animals, dogs, hunters, workers at slaughterhouses. In addition, there are still risks of the population infestation posed by eating raw meat dishes or contacting with the faeces of an animal infected with helminths through untreated berries, leaves, etc. For example, in February 2019, an outbreak of alveococcosis was observed in the Ulyanovsk region among students of the Suvorov school (26 affected people).

The purpose of this research is to study the distribution and extent of cestodiasis affecting domestic reindeer on reindeer herding farms in the Murmansk Oblast, as well as to determine the extent of echinococcosis affecting definitive hosts (domestic and wild carnivores).

MATERIALS AND METHODS

Scientific research was carried out in the reindeer breeding laboratory of the Murmansk State Agricultural Research Station together with the University of Helsinki and the Evira Food Safety Agency (Finland). We studied the morphological and genetic diversity of cestodes in the reindeer meat and internal organs on reindeer farms in the Murmansk Oblast.

The tests were performed in 2018–2019 during the routine slaughter of reindeer at slaughter houses located in the settlements Lovozero, Krasnoschelye, Sosnovka. A total of 4,048 carcasses of domesticated reindeer were examined: Lovozero – 2,812, Sosnovka – 396, Krasnoschelye – 840. During the meat inspection, the parenchymal organs were examined for cestode cysts, upon detection of which the samples were collected, and gross preparations were prepared from them according to the methods generally accepted in parasitology. The helminth species were determined at the Department of Veterinary and Biosciences, Faculty of Veterinary Medicine, University of Helsinki (Finland).

Sampling of stray and reindeer herding dog faeces was carried out during the routine slaughter of reindeer on the territory of the slaughterhouses of the APC "Tundra" and the APC HRE SEN "Olenevod", and the samples were also collected from pastures and at campsites. A total of 83 samples of dog faeces were collected. One wolverine was subjected to a complete helminthological dissection of some organs according to the Academician K. I. Skryabin's method (1928).

The collected faeces samples were placed in a plastic container and during the working day were delivered to the Murmansk Regional Veterinary Laboratory for microscopic examinations using Fyulleborn and Kotelnikov – Khrenov methods.

RESULTS AND DISCUSSION

During the meat inspection, the parenchymal organs were examined and cestode cysts were sampled. Totally 56 cyst samples were collected, 99% of which were found in the liver tissue. Only one sample was collected in the lung. The results are shown in Table 1.

In APC HRE SEN "Olenevod" at the slaughter house in s. Krasnoschelye 25 cyst samples were collected. In ten samples, *Taenia hydatigena* scolexes were detected, indicative of cysticercosis. At the slaughter house in s. Sosnovka 11 cyst samples were collected. No cestodes were detected.

In the APC "Tundra": at the slaughter facility in s. Lovozero in herds No. 1, No. 2, and No. 8 twenty cyst samples were collected. *Echinococcus canadensis,* causing echinococcosis, was detected in one sample, and *Taenia hydatigena* scolexes, which cause cysticercosis, in fourteen samples.

When assessing the research results presented in Table 1, it can be noted that *Taenia hydatigena* mainly affected the liver, and pulmonary echinococcosis was diagnosed only in one case.

Up to 50% of the samples collected were not helminth cysts. These were liver tissues with fibrosis, scarring and cystic lesions. Most likely, such tissue lesions indicate earlier liver damage caused by different helminth species as well.

Besides, microscopic *Setaria tundra* larvae were found on the surface of a number of cysts. It can be assumed that damages on the surface of the deer's liver, in the form of various types of lesions and scaring, are caused by the migration of *Setaria tundra* larvae from the liver blood flow into the abdominal cavity. In addition, when examining carcasses and collecting samples from internal organs, mesentery, body cavities of some killed deer, adult helminths at pubertal stage were found (Fig.). These nematodes are transmitted by blood-sucking insects – mosquitoes. Considering that the summer of 2018 was very hot for the Murmansk Oblast (in July, on some days, the daytime temperature reached 29–31 °C), the spread of this helminth among reindeer may have accelerated.

As a result of examination of domestic reindeer on reindeer farms in the Murmansk oblast, such helminthic diseases as echinococcosis and cysticercosis were detected. It was determined that only the liver was affected, where the larvae cysts parasitize. In the APC "Tundra" echinococcosis was detected in 0.04% of reindeer, cysticercosis – in 0.5%. In the APC HRE SEN "Olenevod" cysticercosis infested 0.81% of reindeer, and echinococcosis was not detected. In general,

Table 1
Results of assessing the epidemic situation of cestodiasis in domestic reindeer on reindeer farms in the Murmansk Oblast

Таблица 1
Результаты оценки эпизоотической ситуации по цестодовой инвазии домашних северных оленей в оленеводческих хозяйствах Мурманской области

Date of testing	Sex and age group	Infested organ	Number of samples	Cyst diameter, mm	Helminth presence	Helminth species
S. Krasnoschelye						
18.02.2019	adult (2–4 y. o.)	liver	2	14–20	Yes	*Taenia hydatigena*
			1	30	Yes	*Setaria tundra*
			2	5	No	–
22.02.2019	adult (2–4 y. o.)	liver	4	5–10	No	–
23.02.2019	adult (2–4 y. o.)	liver	1	5	Yes	*Taenia hydatigena*
			4	5–8	No	–
04.03.2019	calves (males)	liver	3	12–22	Yes	*Taenia hydatigena*
26.03.2019	adult (2–4 y. o.)	liver	4	13–30	Yes	*Taenia hydatigena*
			2	7–15	Yes	*Setaria tundra*
			2	5–15	No	–
s. Sosnovka						
13.01.2019	calves	liver	2	3	No	–
14.01.2019	calves	liver	4	4–10	No	–
16.01.2019	calves	liver	3	4–21	No	–
17.01.2019	calves	liver	2	3–4	No	–
s. Lovozero						
19.12.2018	calves	lung	1	25	Yes	*Echinococcus canadensis*
27.12.2018	calves	liver	1	15	No	–
09.01.2019	calves	liver	1	30	Yes	*Taenia hydatigena*
			2	4	No	–
10.01.2019	calves	liver	2	15–20	Yes	*Taenia hydatigena*
			1	3	No	–
14.01.2019	calves	liver	1	12	Yes	*Taenia hydatigena*
			1	13	No	–
22.01.2019	calves	liver	2	13–15	Yes	*Taenia hydatigena*
23.01.2019	calves	liver	1	10	Yes	*Taenia hydatigena*
12.02.2019	calves	liver	2	10–12	Yes	*Taenia hydatigena*
14.02.2019	calves	liver	5	10–22	Yes	*Taenia hydatigena*

25 animals were infested with cestodes on reindeer herding farms, which amounted to 0.62% of the tested domestic reindeer, of which echinococcosis was diagnosed in 0.02% of cases, and cysticercosis – in 0.59% (Table 2).

According to the information of the Rospotrebnadzor Administration for the Murmansk Oblast, only three cases of echinococcosis in humans were registered in the region from 2010 to 2017. At the same time, the last time this

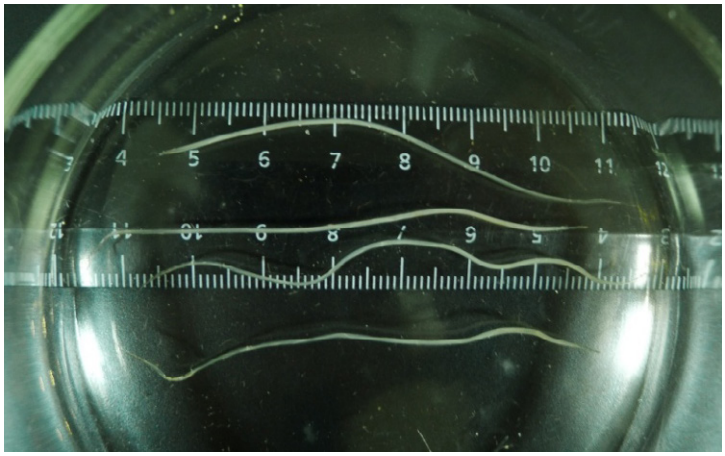

Fig. (Рис.) Setaria tundra

disease was observed twice in 2015. No cases of cysticercosis were reported in humans.

A similar situation with echinococcosis is observed in Finland. Every year, when examining the meat of wild reindeer, the country's Veterinary Service (Evira) detects isolated cases of echinococcus infestation. In 2015, one case of a child infested with echinococcosis was reported [17, 18].

According to the results of the microscopic examination of the faeces from carnivores of the APC "Tundra" *Echinococcus* (*Echinococcus granulosus*) eggs were not found. Nematode eggs were found in dogs of various types of keeping. The extensiveness of invasion in dogs was 28%, in case of helminthological dissection of a wolverine – 100% (Table 3).

As a result of microscopic examination no *Echinococcus* (*Echinococcus granulosus*) eggs were found in carnivores in the APC HRE SEN "Olenevod". Nematode eggs were found in dogs of various types of keeping. The extensiveness of infestation was 49% (Table 4).

CONCLUSION

According to the results of testing of internal organs (liver and lungs) from 4,048 domestic reindeer for cestode cysts, the extensiveness of echinococcal and cysticercosis infestation was determined. On deer farms of the Murmansk Oblast cysticercosis was mainly detected in the liver of domestic reindeer. Extensiveness of echinococcal invasion was 0.02% in the entire tested deer population, cysticercosis – 0.59%. It is generally known that the degree of infestation of deer with echinococcus larvae primarily depends on the quality and frequency of de-worming treatment of reindeer herding dogs. Examination of dogs in direct contact with reindeer and slaughter products showed the presence of nematodes. No cases of cestodosis were observed in the examined animals.

Currently, no effective measures have been developed in reindeer husbandry to combat the helminth cysts. Therefore, the main preventive measures should be aimed at de-worming treatment of dogs with the subsequent destruction of faeces, as well as condemnation and destruction of the affected parenchymal organs and tissues of deer during slaughter. Considering that, in general, the situation in the oblast in terms of the incidence of cestodosis among people, including those directly employed in the field of reindeer husbandry, is quite favorable compared to other regions of the Russian Federation, for example, the Yamalo-Nenets Autonomous Okrug [19], it can be stated that measures to prevent the spread of helminthiasis among domestic and farm animals are performed quite effectively. Our closest neighbors in the Arctic region also have a great influence on the helminthiasis situation.

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Table 2
Indicators of the extensiveness of invasions caused by the cestodes *Taenia hydatigena* and *Echinococcus canadensis* in domestic reindeer at the APC HRE SEN "Olenevod" and APC "Tundra"

Таблица 2
Показатели экстенсивности инвазий, вызываемых цестодами *Taenia hydatigena* и *Echinococcus canadensis*, у домашних северных оленей в СХПК ОПХ МНС «Оленевод» и СХПК «Тундра»

Farm	Testing date	Slaughter facility, herd number	Number of tested carcasses	Number of infested carcasses		Extensiveness of infestation %	
				cysticercosis	echinococcosis	cysticercosis	echinococcosis
APC HRE SEN "Olenevod"	January, 2019	s. Sosnovka, No. 3	396	–	–	–	–
	February, 2019	s. Krasnoschelye, No. 2	364	3	–	0.82	–
	March, 2019	s. Krasnoschelye, No. 2–3	476	7	–	1.47	–
		Total	1,236	10	–	0.81	–
APC "Tundra"	December, 2018	s. Lovozero, No. 1	617	–	1	–	0.16
	January, 2019	s. Lovozero, No. 2, 8	1,207	7	–	0.58	–
	February, 2019	s. Lovozero, No. 1, 8	988	7	–	0.71	–
		Total	2,812	14	1	0.50	0.04
TOTAL			4,048	24	1	0.59	0.02

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Table 3
Indicators of the extensiveness of helminth-associated invasions in deer-herding, hunting and stray dogs in APC "Tundra", Lovozersky Raion, Murmansk Oblast

Таблица 3
Показатели экстенсивности инвазий, вызываемых гельминтами, у оленегонных, охотничьих и бродячих собак в СХПК «Тундра» Ловозерского района Мурманской области

Carnivore species	Number of tested faeces	Number of detected helminth eggs		Extensiveness of infestation, %	
		cestodes	nematodes	cestodes	nematodes
Deer-herding dogs	19	–	3	–	15.8
Hunting dogs	11	–	2	–	18.2
Stray dogs	6	–	5	–	83.3
Wolverine	1	–	1	–	100.0
Total	37	–	11	–	29.7

Table 4
Indicators of the extensiveness of helminth-associated invasions in deer-herding, hunting and stray dogs in APC HRE SEN "Olenevod", Lovozersky Raion, Murmansk Oblast

Таблица 4
Показатели экстенсивности инвазий, вызываемых гельминтами, у оленегонных, охотничьих и бродячих собак в СХПК ОПХ МНС «Оленевод» Ловозерского района Мурманской области

Carnivore species	Number of tested faeces	Number of detected helminth eggs		Extensiveness of infestation, %	
		cestodes	nematodes	cestodes	nematodes
Deer-herding dogs	27	–	9	–	33.3
Hunting dogs	5	–	1	–	20.0
Stray dogs	15	–	13	–	86.7
Total	47	–	23	–	48.9

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Visualization of microbial biofilms in case of digestive disorders in lambs

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SUMMARY

The paper demonstrates morphometric and densitometric parameters of microbial biofilms recovered from lambs with digestive disorders. Changes of quantitative and species composition of the intestinal microbiocenoses in the lambs with digestive disorders were compared with the ones of the clinically healthy lambs. Light microscopy results demonstrated formation of three-dimensional biofilm structure in the form of dense grid consisting of gram-negative and gram-positive bacteria, yeast cells, hyphas and pseudohyphas surrounded with intracellular polymer matrix. Presence of blastospores aided to the increased number of cells attached to the substrate, and biofilm was formed, which consisted of rod and round cells attached to the microfungi cells. In the process of dispersion that occurred during the destruction of the intercellular matrix and bacterial and yeast cell detachment, branched structures separated from the microcolonies and colonized microorganism-free regions of the substrate. The intensity of biofilm formation by the microorganisms under study was evaluated by optic density measurement in 48 hours of cultivation. Fluorescence microscopy results demonstrated that the dynamics of changes of the viable microbial structures was specified by intermittent periods of increased or decreased biofilm formation intensity. Cells characterized by active growth and replication and forming alternating subpopulations were detected in the examined microbial cultures. When determining the viability of the microorganisms in the biofilms, the viable (green fluorescence) and non-viable (red fluorescence) cells were differentiated.

Keywords: Attachment, biofilms, bacteria, dispersion, microfungi, colonization resistance, intercellular matrix, fluorescence.

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Индикация биопленок микроорганизмов при болезнях органов пищеварения ягнят

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РЕЗЮМЕ

Приведены результаты морфометрических и денситометрических показателей биопленок микроорганизмов, выделенных при болезнях органов пищеварения ягнят. Установлены изменения количественного и видового состава микробиоценозов кишечника при болезнях органов пищеварения по сравнению с клинически здоровыми ягнятами. При проведении исследований методами оптической микроскопии выявили формирование трехмерной структуры биопленок в виде плотной сети, состоящей из грамотрицательных и грамположительных бактерий, дрожжевых клеток, гифальных и псевдогифальных форм, окруженных межклеточным полимерным матриксом. Наличие бластоспор обусловливало увеличение количества адгезированных к субстрату клеток, происходило формирование биологической пленки, состоящей из палочковидных и округлых клеток, удерживающихся на клетках микроскопических грибов. В процессе дисперсии при разрушении межклеточного матрикса и отделении бактериальных и дрожжевых клеток от микроколоний обособлялись разветвленные структуры и колонизировали свободные от микроорганизмов участки субстрата. Оценку интенсивности формирования биопленок изучаемыми микроорганизмами проводили, измеряя оптическую плотность через 48 ч культивирования. Результаты люминесцентных микроскопических исследований показали, что динамика изменений жизнеспособных структур микроорганизмов характеризовалась чередующимися периодами снижения и увеличения интенсивности формирования биопленки. В исследуемых культурах микроорганизмов выявляли клетки, характеризующиеся способностью к активному росту и размножению, образующие сменяющие друг друга субпопуляции. При детекции жизнеспособности микроорганизмов в составе биопленок дифференцировали жизнеспособные (зеленый спектр люминесценции) и нежизнеспособные клетки (красный спектр люминесценции).

Ключевые слова: Адгезия, биопленки, бактерии, дисперсия, микроскопические грибы, колонизационная резистентность, межклеточный матрикс, люминесценция.

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INTRODUCTION

The complex of the processes involving microbial circulation and reservation in the soil and water biocenoses is the ecological pillar of enzootic disease emergence as well as formation of the infectious disease agents' foci and their spread to the new territories and water areas [1–2].

Gastrointestinal and respiratory diseases amount up to 95.0% within the structure of the neonatal pathology, and *Salmonella* spp. и *Pasteurella* spp. are their etiological agents [3]. In case of pathologies clinically manifested with diarrhea, dehydration, toxaemia, specifically in case of colibacillosis, the lamb morbidity reaches 12.3–95.2% and lethality – 60.0–90.0% [4, 5]. In colostrum- and milk-suckling lambs decrease of bifid- and lactobacteria concentration and increase of the toxicogenic enterobacteria, enterococci and *Candida* microfungi were reported [6–11]. Small intestine colonization with pathogenic microorganisms, primarily with gram-negative enterobacteria, is associated with morphofunctional properties of the digestive organs of the animals in early postnatal life [12–14].

Incidence of drug resistance of the microorganisms, isolated from pathological material and contents of the purulent lesions from animal skin and muscles is reported [15–18].

Contamination with the microorganisms demonstrating high enzymatic activity, *inter alia*, at low temperatures, can occur at all stages of the food raw material handling and storage thus making the problem socially significant [12, 19, 20].

Pathogenesis of small bowel bacterial overgrowth syndrome following microorganism translocation and occurrence of microbial virulence factors is assured through the attachment properties, which are among the key factors of the biofilm architectonics formation specified by the optic density growth thus determining the length and retrospective properties of the diagnostic tests [21–23]. Biofilms of multiresistant strains of *Candida parapsilosis* demonstrated dissociation processes; densitometric values of the concentric phenotype 1.75 times increased optic density values of "crater" phenotype; optic density parameters of the smooth colonies were 20.0–60.0% lower as compared to other phenotypes [24]. The populations affected by the adverse factors were heterogenic, and they were not genetically different from the original population; they also preserved their genetic diversity necessary for colonization of the "cleansed" niche or new habitat [21, 23, 25, 27].

High performance of instrumental analytical methods was demonstrated for the *in vitro* and *in vivo* detection of viable microorganisms in the heterogenic population, as they allow for the detection of the polysaccharidic intercellular matrix and metabolically active cells through the interaction between the stains and cell wall polysaccharides [28–30].

Phase-contrast microscopy aids to the detection of aggregated motile cells, processes of the cell aggregation, evaluation of the degree of the biofilm biomass gain at different stages of their formation depending on the cultivation medium composition and oxygen content [31].

Electron microscopy of the biofilms of gram-negative, gram-positive bacteria and microfungi enables recovery of the mechanisms of the intercellular communication, sorption and aggregation of heterogenic biofilms as well as cyclic growth mode and phenotypic flexibility that precondition the persistence of nonculturable organisms in the bacteria carriers during the interepidemic periods [12, 21, 23, 32].

In order to reveal the mechanisms of the pathogen adaptation to parasitizing in the susceptible species, optimization of microbiological study design, development of anti-epidemic measures aimed at the animal disease prevention, approbation and selection of the microbial biofilm study tools and methods are of the highest priority thus defining the relevance of this research.

The research was aimed at the examination of morphometric and densitometric properties of microbial biofilms in case of gastrointestinal disorders in lambs.

MATERIALS AND METHODS

Strains. Isolates recovered from the gut contents of Agin breed-lambs at the age from 1 to 30 days old. Before testing, the animals were by analogy subdivided into two groups: Group I – lambs with digestive disorders (experimental, $n = 5$); Group II – clinically healthy lambs (control, $n = 5$).

Nutrient media: brain heart infusion broth, Chromocult® Coliform Agar, Cetrimide Agar, Yolk Salt Agar, HiCrome Candida Agar, Oxytetracycline Glucose Yeast Extract Agar, (HiMedia, India).

Test-kits: ENTERO-Rapid, NEFERM test 24 (Erba-Lachema, Czech Republic), API Staph (bioMérieux, France), Hi-Candida FGP Armavir Biofactory (Russia), RUP Institute of Experimental Veterinary Medicine named after S. N. Vyshelessky (Belarus).

Indication and identification of microorganisms. Morphological, cultural and biochemical properties of the microorganisms were examined using routine methods according to the Bergey's Manual of Systematic Bacteriology (1984–1989) and Guide to clinically significant fungi [33, 34]. The microorganisms were cultivated at 37 °C for 24 hours using liquid and solid nutrient media. *Escherichia* were serologically differentiated using diagnostic sera, and adhesive antigens K88, K99, 987P, F41, A20 were identified according to the "Guide on Use of Agglutinating O-coli sera" (Moscow, 1998).

Biofilm densitometric parameters. Microbial biofilms were indicated by the degree of crystal violet binding (HiMedia, India) at 490 nm wavelength. The tested samples were added to the wells of 96-well plate (OAO "Medpolymer Company", Russia), cultivated in the constant aerobic environment at 37 °C for 48 hours. The liquid was discarded and the wells were washed with 200 µl of phosphate-buffered solution (PBS) for three times (pH 7.3). The plates were shaken for 5 min at each stage of washing. The samples were fixed with 150 µl of 96% ethanol for 15 min and dried out at 37 °C for 20 min. The microbial biofilms were stained by adding 0.5% stain solution in each well and subsequent cultivation at 37 °C for 5 min. The contents of the wells were discarded; the plates were washed with 200 µl of PBS three times (pH 7.3) and dried out. The bound stain was eluted from the attached cells with 200 µl of 96% ethanol for 30 min [35, 36].

Biofilm morphometric parameters. Before the light microscopy, the microorganisms were cultivated at 37 °C for 18–48 hours. The microorganisms were cultivated on the slides placed in the Petri dishes containing 20 ml of pepted meat broth (PMB) and 5 ml of 18-hour culture suspension at 10^5 CFU/ml [21]. Using Gram stain kit (BioVitrum, Russia), the preparations were fixed with ethanol and ether mixture (1:1) for 10 min, stained with 0.5% methylene blue solution and 1:2000 gentian violet water solution. 18.0 × 18.0 mm slides (Corning Inc., USA) were used for fluorescence microscopy. Before inoculation, the slides were rinsed with 70% ethanol / 30% deionized water and dried at 70 °C for 30 min. The slides were then placed in the wells of 12-well plates (OAO "Medpolymer", Russia), and microbial suspension ($OD_{600} = 0.08$) was pipetted at 5.0 ml/well and cultivated at 37 °C for 18–48 hours. The wells were washed with PBS (pH 7.2) twice and dried out. Hereafter, 15 µl of Live/Dead stain was added onto the slides at concentration of 1 mg/ml; the slides were covered with cover slip and stained for 10 min at 25 °C in the dark place [37]. The microscopy was carried out at representative sample with significant frequency ≥ 90.0% of Biomed MS-1 light microscope FOV (OOO "Biomed", Russia); fluorescence microscope Leica DMRB (Germany), equipped with 100× oil immersion objective with 510 nm dichroic filter and 515 nm long-wave pass filter.

The experimental results were statistically processed using Student's t-test and were deemed true at $p \leq 0.05$.

RESULTS AND DISCUSSION

Indication and identification of microorganisms. Lambs with gastrointestinal disorders demonstrated increased levels of bacteria and yeast-like fungi, which formed colonies on differential and diagnostic media: Chromocult® Coliform Agar, Cetrimide Agar, Yolk Salt Agar, HiCrome Candida Agar (Table 1).

By contrast to the clinically healthy animals (control), increased numbers of microorganisms isolated from lambs with gastrointestinal disorders (experimental) were observed on the above-mentioned media as compared with.

Due to sodium dodecyl sulfate present in Chromocult® Coliform Agar medium, growth of gram-positive bacteria was inhibited and growth of gram-positive bacteria increased: experimental – 7.15 ± 0.12 – 9.33 ± 0.26; control – 3.13 ± 0.12 – 7.13 ± 0.10; colonization index (CI) – 0.831%.

During the differentiation of the microorganisms, we bore in mind that in the above mentioned differentiation and diagnostic medium the *Escherichia* formed the colonies of violet color due to the presence of *β*-galactosidase and *β*-glucuronidase enzymes, which simultaneously degraded two chromogenic substrates. *Proteobacteria* and *Enterobacteriaceae* lack these enzymes and they formed colorless colonies on the medium. *Klebsiella* formed dark-pink colonies due to degradation of chromogenic substrate by *β*-galactosidase (Fig. 1a).

Presence of tryptophan in the media allowed testing indole formation by adding Kovack's reagent to the violet colonies. If the colonies changed their color to pink-red within 3–5 sec, the test deemed positive and *Escherichia* could be differentiated from the taxonomically similar *Enterobacteriaceae* spp. within 24 hours.

Enterobacteriaceae were catalase-positive, oxidase-negative and they fermented D-glucose and polyatomic alcohols forming acid and gas. *Escherichia* formed indole, utilized sodium acetate; they did not form hydrogen sulfide or utilize citrate and sodium malonate; they did not produce urease, phenylalanyldeaminase but fermented

Table 1
Quantitative composition of microorganisms in case of digestive disorders

Таблица 1
Количественный состав микроорганизмов при болезнях органов пищеварения

Media	Amount of microorganisms (CFU, lg/g)		Colonization index*, %
	Control	Experiment	
Chromocult® Coliform Agar ($n = 20$)	3.13 ± 0.12 – 7.13 ± 0.10	7.15 ± 0.12 – 9.33 ± 0.26	0.831
Cetrimide Agar ($n = 6$)	1.01 ± 0.12 – 2.01 ± 0.10	2.84 ± 0.11 – 3.03 ± 0.16	0.356
Yolk Salt Agar ($n = 6$)	0.21 ± 0.11 – 0.82 ± 0.10	0.83 ± 0.07 – 1.36 ± 0.09	0.253
HiCrome Candida Agar ($n = 6$)	1.74 ± 0.13 – 2.18 ± 0.03	4.14± 0.12 – 5.01 ± 0.08	0.420

* Proportion of microorganisms (CFU, lg/g) in 1.0 g of the tested sample collected from clinically healthy animals (control) and microorganisms (CFU, lg/g) in 1 g of the tested sample collected from the animals with digestive disorders (experimental).

* Отношение количества микроорганизмов (КОЕ, lg/г) в 1,0 г исследуемого материала клинически здоровых животных (контроль) и количества микроорганизмов (КОЕ, lg/г) в 1,0 г исследуемого материала при болезнях органов пищеварения (опыт).

sucrose and dulcite. *Klebsiella* utilized glucose, sodium citrate and produced acetylmethylcarbinol; they fermented inose and hydrolyzed urea, but did not form indole and hydrogen sulfide. *Proteobacteria* formed hydrogen sulfide and urease; they reduced nitrates, hydrolyzed gelatin, fermented glucose and demonstrated positive reaction with methyl red; they also deamidated phenylalanine, failed to decarboxylize lysine and differed in their ability to utilize sodium citrate. *Enterobacteriaceae* did not form indole and hydrogen sulfide; they fermented glucose, lactose, rhamnose, xylose, maltose, sorbite, arabinose, raffinose; they utilized citrate, sodium malonate; hydrolyzed gelatin and differed in their ability to utilize inose, dulcite, salicin and adonitol (Table 2).

During the recording of differentiating features the following bacteria were identified among the twenty isolated gram-negative pure microbial cultures: 19 *Enterobacteriaceae* isolates (95.0%), of which 13 (65.0%) were *Escherichia coli*, 4 (20.0%) – *Klebsiella pneumonia*, 2 (10.0%) – *Proteus vulgaris* and 1 (5.0%) – *Enterobacter cloacae*.

Serological tests of 13 (65.0%) identified *E. coli* isolates demonstrated that three isolates were positive for polyvalent Group 1 serum (serogroups O2, O78, O33); four (20.0%) – for Group 2 serum (serogroups O9, O15, O26, O111), two (10.0%) – for Group 3 serum. Four microbial cultures produced adhesive antigens: O33:F41 – 1 (5.0%), O111:A20 – 1 (5.0%), O2:A20 – 1 (5.0%), O9:A20 – 1 (5.0%).

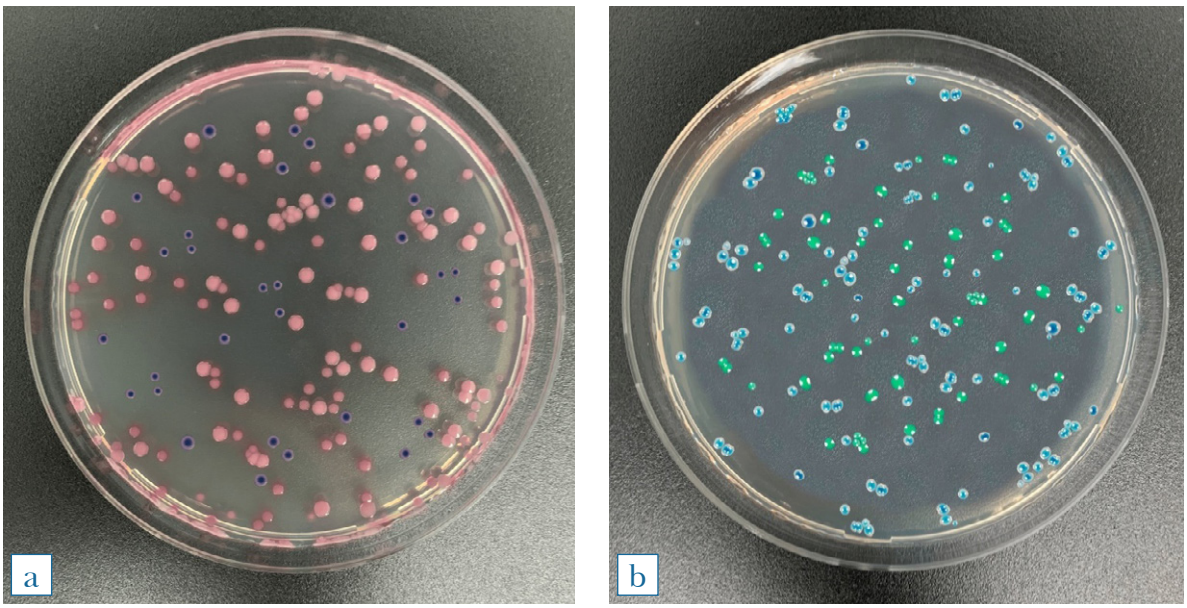

Fig. 1. Morphology of microbial colonies in intestinal microbiocinoses of lambs with digestive disorders (growth at 37 °C, 48 hours):
a – Chromocult® Coliform Agar, 76 × 10^{-9} CFU; b – HiCrome Candida Agar, 68 × 10^{-9} CFU

Рис. 1. Морфология колоний микроорганизмов микробиоценозов кишечника ягнят при болезнях органов пищеварения (рост при 37°С, 48 ч):
a – Chromocult® Coliform Agar, 76 × 10^{-9} КОЕ; b – HiCrome Candida Agar, 68 × 10^{-9} КОЕ

Increased amounts of microorganisms in case of digestive disorders were also observed on Cetrimide Agar: experimental – 2.84 ± 0.11 –3.03 ± 0.16; control – 1.01 ± 0.12 – 2.01 ± 0.10; colonization index – 0.356%. Out of six recovered microorganisms, one isolate (16.7%) was identified as gram-negative aerobic *Pseudomonas aeruginosa*. The above-mentioned bacteria converted nitrites to nitrates, they possessed proteolytic properties (dissolved gelatin and coagulated blood sera, hydrolyzed casein), coagulated litmus milk and broke down clots; they did not ferment maltose or form indole and hydrogen sulfide.

The number of microorganisms also increased on Yolk Salt Agar that contained 10.0% of sodium chloride: experimental – 0.83 ± 0.07 – 1.36 ± 0.09; control – 0.21 ± 0.11 – 0.82 ± 0.10; colonization index – 0.253%. Out of six recovered microorganisms, two isolates (33.3%) were identified as gram-positive *Staphylococcus* spp.: *S. aureus* – 1 (16.7%), *S. epidermidis* – 1 (16.7 %). These bacteria formed dome-shaped nontransparent colonies of white, golden, orange or yellow color and 2.0–2.5 mm in diameter. Coagulation of blood serum plasma was reported, which contained 1.0–4.0% of sodium citrate and clotted on the slide. Growth was observed with 15.0% of sodium chloride or 40% of bile. Under anaerobic conditions, the microorganisms fermented glucose and mannitol; they produced ammonia, coagulated and peptonized milk, and they did not ferment dulcite, salicin and inulin.

The number of microbial colonies on HiCrome Candida Agar also increased: experimental – 4.14 ± 0.12 – 5.01 ± 0.08; control – 1.74 ± 0.13 – 2.18 ± 0.03; colonization index – 0.420%. Out of six recovered microorganisms, three isolates (50.0%) were identified as yeast-like fungi: *Candida albicans* – 2 (33.3%), *Candida parapsilosis* – 1 (16.7%). The microorganisms grown on Sabouraud glucose agar formed smooth, dome-shaped colonies of white color and soft, even consistency (S-shape). Due to the degradation of chromogenic hexoaminidase substrate with β-N-acetylgalactominidase, *C. albicans* microfungi formed the colonies of pale green color. *C. parapsilosis* colonies, lacking the above-specified enzyme, formed blue colonies on the medium (Fig. 1b).

The yeast-like fungi *Candida* spp. could grow with cycloheximide; they fermented sucrose, maltose, xylose and had no urease activity. *C. albicans* fermented galactose and *C. parapsilosis* did not ferment trehalose (Table 3).

Densitometric parameters of microbial biofilms. The tested microorganisms were distributed by the intensity of biofilm formation according to optic density measurements in 48 hours of cultivation.

The obtained results demonstrated that absolute values of optic density (OD_s) of *P. aeruginosa*, *S. aureus* and *C. albicans* amounted to 0.454 ± 0.09 – 0.526 ± 0.08, biofilm formation intensity – $I \geq 0.3–0.4$; hence, these are powerful biofilm-producing microorganisms.

Tests for biofilm formation capacities of *E. coli*, *K. pneumonia*, *C. parapsilosis* demonstrated that their optic density (OD_s) ranged from 0.391 ± 0.07 to 0.571 ± 0.05, and biofilm formation intensity was $I \geq 0.2–0.3$. Therefore, these microorganisms were categorized as moderate biofilm-producing microorganisms.

It was determined that *P. vulgaris* and *E. cloacae* had poor capacities of biofilm formation: their optic density (OD_s) ranged from 0.246 ± 0.03 to 0.284 ± 0.08 and biofilm formation intensity was $I \geq 0.1–0.2$ (Table 4).

Morphometric parameters of microbial biofilms. In 18–48 hours of cultivation at 37 °C, microscopy of gentian violet-,

Table 2
Differentiation of enterobacteria by their biochemical properties

Таблица 2
Дифференциация энтеробактерий по биохимическим свойствам

Biochemical parameters	Bacterium species			
	Escherichia coli	*Klebsiella pneumoniae*	*Proteus vulgaris*	*Enterobacter cloacae*
Oxidase	–	–	–	–
Catalase	+	+	+	+
Lactose	+	+	–	+
Indole	+	–	+	–
Sorbitol	+	+	–	+
Hydrogen sulfide	–	–	+	–
Citrate	–	+	+	+
Urea	–	+	+	–
Gelatin	–	–	+	+
Voges-Proskauer reaction	–	+	+	+

"+" – positive test result (положительный тест);
"–" – negative test result (отрицательный тест).

Table 3
Differentiation of yeast-like fungi *Candida* spp. by their biochemical properties

Таблица 3
Дифференциация дрожжеподобных грибов *Candida* spp. по биохимическим свойствам

Carbohydrate	Microorganism	
	C. albicans	*C. parapsilosis*
Urease	–	–
Melibiose	–	–
Lactose	–	–
Maltose	+	+
Sucrose	+	+
Galactose	+	–
Cellobiose	–	–
Inose	–	–
Xylose	+	+
Dulcite	–	–
Raffinose	–	–
Trehalose	+	–

"+" – positive test result (положительный тест);
"–" – negative test result (отрицательный тест).

Table 4
Determination of the bacterial biofilm formation intensity by optic density

Таблица 4
Оценка интенсивности формирования биопленок бактериями по оптической плотности

Microbial cultures	Cell size, nm	Optic density				
		OD_s			OD_c	t_d
		1	2	3		
E. coli	(1.4–3.8) × (0.5–0.8)	0.542	0.571	0.550	0.098	4.2
K. pneumoniae	(0.6–6.0) × (0.3–1.0)	0.514	0.493	0.502	0.099	4.4
P. vulgaris	(1.0–3.0) × (0.4–0.8)	0.284	0.279	0.275	0.097	4.2
E. cloacae	(0.6–1.0) × (1.2–3.0)	0.246	0.256	0.267	0.099	3.6
P. aeruginosa	(1.5–5.0) × (0.5–1.4)	0.458	0.462	0.454	0.098	3.9
S. aureus	(1.5–1.6)	0.481	0.485	0.477	0.098	4.4
C. albicans	(2.0–3.0) × (3.0–5.0)	0.520	0.532	0.526	0.099	4.5
C. parapsilosis	(1.7–2.0) × (3.0–4.0)	0.391	0.397	0.403	0.097	4.2

OD_s – tested sample (исследуемый образец); OD_c – control (контроль); t_d – confidence factor (коэффициент достоверности).

methylene blue- and Gram-stained preparations demonstrated heterogeneous structures of the biofilms formed by the tested isolates of the gram-negative and gram-positive bacteria and yeast-like fungi *Candida* spp. (Fig. 2).

Intercellular communication processes were implemented stepwise: sedimentation; fixation (primary attachment); formation of monolayer and intercellular connections (co-aggregation), growth of microcolonies; formation of clusters and architectonics of the mature biofilms; dispersion.

During the destruction of intercellular matrix and detachment of the bacterial and yeast cells from the microcolonies, the dispersion was detected as separate arm-like structures, which colonized those areas of the substrate that were free from the microorganisms.

Through the synthesis of the intercellular polymer matrix, the 3D-structure of biofilms is formed as a dense grid of gram-negative and gram-positive bacteria, yeast cells, hyphas and pseudohyphas. Multi-species biofilms were generally characterized by the attachment of

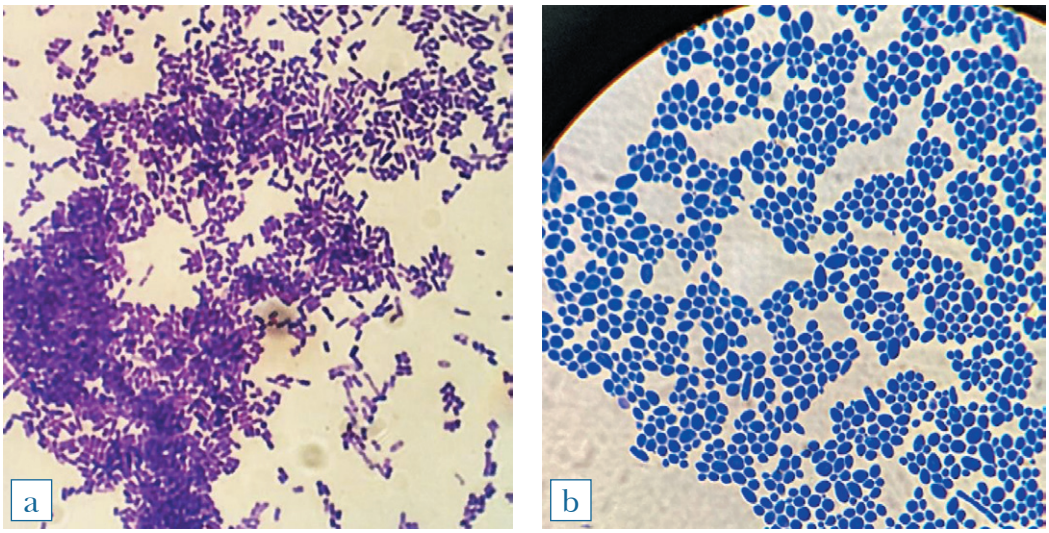

Fig. 2. Morphology of microbial biofilms (growth at 37 °C, 48 hours, MPB):
a – K. pneumoniae bacteria. Staining with methylene blue, oc. 10, obj. 100, immersion;
b – yeast-like fungi C. parapsilosis. Staining with methylene blue, oc. 10, obj. 100, immersion

Рис. 2. Морфология биопленок микроорганизмов (рост при 37 °С, 48 ч, МПБ):
a – бактерии K. pneumoniae. Окрашивание метиленовым синим, ок. 10, об. 100, иммерсия;
b – дрожжеподобные грибы C. parapsilosis. Окрашивание метиленовым синим, ок. 10, об. 100, иммерсия

gram-negative and gram-positive bacteria to the yeast-like fungi. Presence of microfungial blastospores aided to the increased number of cells attached to the cellular substrate, and biofilm was formed, which consisted of bacterial cells attached to the yeast cells (Fig. 3).

In 18–72 hours of cultivation, the fluorescence microscopy demonstrated that the dynamics of changes of the viable microbial structures was specified by intermittent periods of increased or decreased biofilm formation intensity. Cells characterized by active growth and replication and forming alternating subpopulations were detected in the examined microbial cultures. When determining the viability of the microorganisms within the biofilms, the viable (green fluorescence) and non-viable (red fluorescence) cells were differentiated. Alternations of the microbial growth intensity are driven by the presence of various dissociative variants advantaging during the formation of the biofilm architectonics. Owing to the reaction of the fluorescent stains with the cell wall polysaccharides, structures of the metabolically active cells were differentiated within the biofilms: green fluorescence – viable cells and red fluorescence – non-viable cells (Fig. 4).

CONCLUSION

Twenty-six isolates were recovered from 38 lambs with gastrointestinal disorders, including: *Escherichia coli* – 13, *Klebsiella pneumoniae* – 4, *Proteus vulgaris* – 2, *Enterobacter cloacae* – 1, *Pseudomonas aeruginosa* – 1, *Staphylococcus aureus* – 1, *Staphylococcus epidermidis* – 1, *Candida albicans* – 2, *Candida parapsilosis* – 1.

In 18–48 hours of cultivation at 37 °C, the light microscopy demonstrated heterogeneous structure of the biofilms formed by the tested isolates of the gram-negative and gram-positive bacteria and yeast-like fungi *Candida* spp.

Owing to the reaction of the fluorescent stains with the cell wall polysaccharides, structures of the metabolically active cells were differentiated within the biofilms: green fluorescence - viable cells and red fluorescence – non-viable cells.

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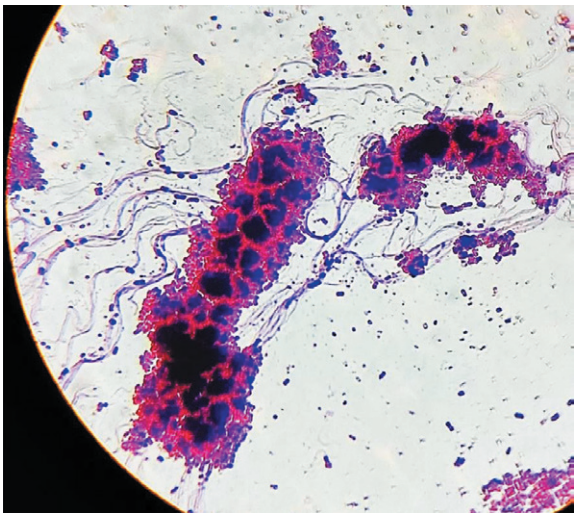

Fig. 3. Morphology of microbial biofilms (growth at 37 °C, 48 hours, MPB): P. aeruginosa bacteria and yeast-like fungi C. albicans. Gram staining, oc. 10, obj. 100, immersion

Рис. 3. Морфология биопленок микроорганизмов (рост при 37 °С, 48 ч, МПБ): бактерии P. aeruginosa и дрожжеподобные грибы C. albicans. Окрашивание по Граму, ок. 10, об. 100, иммерсия

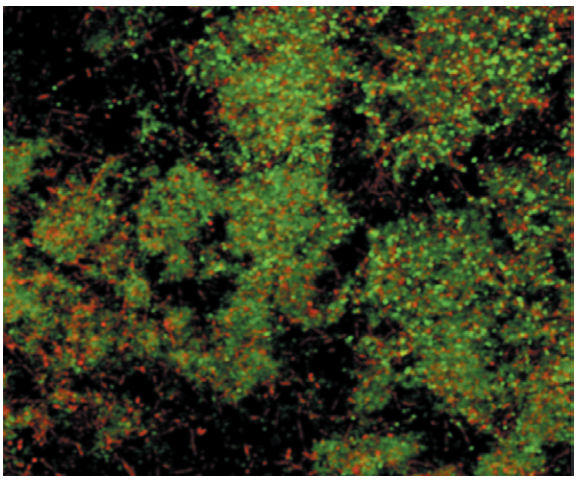

Fig. 4. Morphology of K. pneumonia biofilms (growth at 37 °C, 72 hours, MPB). Live/Dead stains, oc.10, obj. 200, immersion

Рис. 4. Морфология биопленок бактерий K. pneumonia (рост при 37 °С, 72 ч, МПБ). Комплекс красителей Live/Dead, ок. 10, об. 200, иммерсия

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Review article: key aspects of mammal microbiome development

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SUMMARY
This review article summarizes current understanding of the microbiota development in neonatal mammals based on the results of modern experimental studies in animals focusing on three aspects: initial colonization, microbiota effect on the immune function of the developing newborn animal intestine and external factors influencing the microbiome shaping during the juvenile period. The presented study results confirm that the microbial landscape correction is the most important factor for animal health improvement since healthy microflora contributes to the intestinal infection frequency and intensity reduction, and this, in turn, minimizes the use of antibiotics. The microbiome is known to have an impact on the immune system development, metabolic processes and even on the ethology, so an atypical microbial population can cause immune and metabolic disorders. The active interaction between microorganisms and the host organism begins already at birth. Even different modes of delivery (caesarean or vaginal delivery) may determine the initial colonization of the newborn. The animal genetics, nutrition and environment also influence the intestinal microbiota development. In this regard, further studies of probiotics are important to understand their efficacy for diarrhea prevention and treatment, their use as an alternative to antibiotics as well as for enhancement of the animal resistance to stress factors.

Keywords: Intestinal microbiome, colonization, mammals, young animals, livestock animals.

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Обзор: ключевые моменты в процессе становления микробиома млекопитающих

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РЕЗЮМЕ
Данный обзор направлен на обобщение современного понимания становления микробиоты новорожденных млекопитающих на основе результатов современных исследований в экспериментах с животными в трех аспектах, включая начальную колонизацию, влияние микробиоты на иммунную функцию кишечника развивающегося новорожденного и воздействие внешних факторов на формирование микробиома в ювенильный период. Результаты представленных в статье исследований подтверждают, что коррекция микробного пейзажа – важнейший фактор улучшения здоровья животного с учетом того, что именно здоровая микрофлора способствует снижению частоты и интенсивности кишечных инфекций, а это, в свою очередь, минимизирует использование антибиотиков. Известно, что микробиом влияет на развитие иммунной системы, метаболические процессы и даже на этологию, в связи с чем нетипичная микробная популяция способна вызвать нарушения как иммунитета, так и метаболизма организма. Активный процесс взаимодействия микроорганизмов и организма хозяина начинается уже при рождении. Даже различные способы родов – кесарево или вагинальное родоразрешение – могут определять начальную колонизацию новорожденного. Кроме того, на формирование микробиоты кишечника влияют генетика особи, питание и окружающая среда. В связи с этим актуально изучение пробиотиков – понимание их эффективности при профилактике и лечении диареи, использование как альтернативы антибиотикам, а также для повышения устойчивости животных к стрессовым факторам.

Ключевые слова: Микробиом кишечника, колонизация, млекопитающие, молодняк, сельскохозяйственные животные.

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INTRODUCTION

On livestock farms, gastro-intestinal pathology in young animals is the most significant economic factor causing financial losses as compared to other diseases. Diarrhea affects the fluid and nutrient absorption that contributes to growth impairment and overall negative effect on the animal health. Furthermore, if not treated promptly, this disorder rapidly spreads in the animal population and can result in high mortality [1].

Currently, diarrhea is commonly prevented with antibiotics against pathogenic bacteria being frequent diarrhea causes. A significant disadvantage of antibiotic use is emerging and spread of antimicrobial-resistant microorganism strains [2]. In large livestock holdings, bacteria rapidly acquire resistance genes due to constant presence of large numbers of animals in the limited space [3]. Therefore, effective and justified alternative for antimicrobial prevention of diarrhea in young livestock is required.

Many studies allow us to consider the gut microbiota as an important factor for the immune function development and the newborn health support. From this point of view, the intestinal microbiome determines the young animal health and resistance to intestinal infections. Nevertheless, the mechanisms of the microbiota impact on the immune and metabolic process development and establishment are largely undetermined [4].

Even Louis Pasteur has suggested that the microbiota has an important effect on the mammals' life. Animals as hosts have abundant and diverse population of microorganisms directly influencing the mechanisms of such biological processes as immunity and metabolism [5].

MICROFLORA EFFECT ON IMMUNE SYSTEM DEVELOPMENT

Host-microflora interaction plays a vital role in appropriate immune system development. Many researchers believe that the critical period for animals starts immediately after their birth when microbial antigen exposure is required for the immunity development [5–7]. Microflora has an impact on the immune system phylogenesis, onthogenetic development patterns of mucosal and general immunity as well as on anti-infective response effectiveness and adequacy. Various disorders during the body microbiota establishment, especially in gastro-intestinal tract, result in immune-dependent diseases [7]. This is confirmed by the studies of the immune system in sterile animals having poorly developed mucosa-associated lymphoid tissue, hypoplastic peyer's patches and characterized by decreasing numbers of CD4+-lymphocytes and IgA-producing plazmacytes in the mucosa lamina propria, and at the same time, by the shift in the T-cell differentiation towards type 2 T-helper cells in the lymphoid organs and decreased γ-interferon production [6, 8].

During the experiment in sterile mice H. Sokol et al. demonstrated that microflora-free mice developed atypical cytokine response to orally administered treated lipopolysaccharide (immune response-activating macromolecule of outer membrane of gram-negative bacteria) as compared to normal mice having gut microflora. Described atypical response differed from normal response in its delayed development followed by excessive cytokine release. In addition, sterile juvenile mice treated with *Bifidobacterium infantis* were found to be capable of return to normal cytokine response whereas in adult mice treated with the same probiotic the immune response failed to normalize. Thus, it was demonstrated that the sterile animals could not resist to pathogens [5]. Importance of own normal microflora development at early age for avoiding immune system malfunctions in future was demonstrated.

In their studies, I. H. Ismail et al. showed that the microbiome disorders in young animals could later result in autoimmune diseases, including allergy. At the same time, the authors demonstrated that the development of these pathological conditions could be minimized by probiotics [9].

FACTOR INFLUENCING MICROBIOTA ESTABLISHING

Currently, newborn mammals are believed to be sterile, and the initial microbial colonization of their bodies can occur during and immediately after their birth, when microbes first interact with the body and colonize it. Since this process is rapid, it is supposed to start in the birth canal or at the first environmental exposure of the newborns in the case of a caesarean section [6, 10, 11].

Contamination of environment objects with pathogenic and opportunistic microorganisms often has a negative impact on the animal microflora development resulting in decrease in obligatory microorganisms in its gastrointestinal biotope. During the experiment in Holstein and black-and-white calves, Russian researchers detected *Bifidobacterium* spp. in 1 g feces (diluted at 10^{-3}–10^{-10}) from clinically healthy animals whereas the said microorganisms were not detected in animals with diarrhea, toxemia and dehydration. In addition, a decrease in lacto-positive *Escherichia coli* strains and increase in lactose-negative enetrobacteria and bacteria with low lactase activity were observed as well as hemolytic *Escherichia coli* strains were detected in diseased animals [12].

In general, microbiota establishment is a dynamic process following the initial colonization that depends on such factors as host genetics, diet, maternal stress, interaction with the environment, early exposure to antibiotics [13].

In particular, the macroorganism genetics determine the intestine microenvironment that, in turn, influences the suitability of the internal environment for microbial colonization. Z. A. Khachatryan et al. detected an apparent association between gene mutations and relevant changes in the intestinal microflora characterized by depletion of the total bacteria number, loss of diversity and significant quantitative changes in the populations of some bacteria [14]. Nevertheless, no genetic association between intestinal microbiota and long-term growth and weight indicators has not been yet detected that requires further investigations in this field.

In addition, prenatal maternal stress could have an impact on the offspring intestinal microflora composition. According to D. Zhou et al., the dam's physiological parameters change during this period including a rapid heartbeat and stress hormone release. Moreover, the dam's behavior may change with increase or decrease in her appetite and activity. Such changes may have an impact on dam's microflora and potentially on the initial colonization of her offspring at birth. Maternal stress can also result in increase in cytokine concentration and, as consequence, in inflammation than affects the developing fetus causing changes in the fetus immune function [15].

MICROBIOTA EFFECT ON ETHOLOGY AND ADAPTATION

N. Sudo et al. presented evidence on the association of microorganisms with host behavioral responses based on the results of comparative tests of sterile mice and mice colonized with bacteria. To test the hypothesis that postpartum microbial colonization may have an impact on the development of the brain plasticity, the researchers compared the hypothalamic-pituitary response at different stress levels in two groups of genetically identical mice. Mice of one group were sterile and mice in other group were colonized with the specific bacteria. During the experiment, the mice were placed in 50 ml conical tube for one hour or in glass container covered with ether-soaked filter paper for 2.5 minutes. Sterile mice demonstrated more intensive stress response as compared to the mice with microflora. Moreover, decreased stress response was observed in the sterile mice following *Bifidobacterium infantis* probiotic administration [16].

In other studies, P. Bercik et al. detected that administration of antimicrobials to the mice colonized by the complete intestinal microbial community impaired the intestinal microbiota composition and enhanced the response to the stress factor. Therewith, the same antimicrobials administered to sterile mice had no impact on their behavior [17]. The study results demonstrated a potential association between intestinal microflora and animal behavior.

Experiment results published by M. Vijay-Kumar et al. showed the microbiota impact on the host metabolic dysfunction. Toll-like receptor 5 (TLR5)-deficient mice were used for the experiments. The said membrane protein is known to play a key role in gut innate immunity and to participate in pathogenesis of obesity-associated chronic inflammation. Then, the microbiota of these mice were inoculated to sterile wild-type mice. Significant hyperphagia and progressing metabolic disorder were observe in tested TLR5-deficient mice. Sterile wild-type mice colonized with the microbial strains derived from the intestines of the TLR5-deficient mice immediately manifested distinct changes in their intestinal microbiota, and then signs of the metabolic syndrome (obesity) [18]. The changes in microbe-free mice body constitution from normal to obese after colonization by the specific microbial population suggests that the gut microbiota could affect the host's metabolic functions.

CONCLUSION

Microbial colonization of young animals has a significant impact on their physiology and performance. The intestinal microflora and its proper development, particularly at a young age, can significantly determine the further functioning of certain systems, as well as act as a potential tool for reducing the diarrhea incidence in young livestock animals [1]. In this regard, studies of probiotics are of the most importance. It is important to understand how effective probiotics are for diarrhea prevention and treatment, whether they can serve as a full-fledged alternative to antibiotics, and whether they can be used to enhance animals' resistance to stress factors. Impact of dam's genetics and body state on her offspring microbiota formation is also important. Given the fact that the intestinal microbial landscape is a very mobile and fragile system, use of antimicrobial drugs for preventive purposes should be avoided.

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OIE and FAO join forces to counter ASF

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SUMMARY

African swine fever (ASF) is an infectious disease of domestic and wild pigs, which went beyond its natural range (African continent) in the XXI century and since 2007 (emergence in Georgia) has spread to many European and Asia-Pacific countries. According to the immediate notifications and follow-up reports, by early 2021 Europe accounted for about 68% of globally reported outbreaks. However, the greatest losses in the pig industry were inflicted by the outbreak recorded in Asia in 2020, when 6,733,791 animals died that accounted to 82% of the total global losses due to ASF. Just after several years of the current ASF epizootic, without any vaccine or treatment available, it became clear that major problems for the pig industry (mostly for small farmers) as well as destabilization of the global market of pig products were unavoidable. In this regard, in 2014 (Bern, September 2014) a regional standing group of experts on African swine fever (SGE ASF) was established under FAO/OIE GF-TADs umbrella. The aim of the group is to foster closer collaboration between the affected countries, increase transparency and share experience in prevention and control. The work of the permanent expert ASF missions under the GF-TADs umbrella has proven effective and become a model for other regions. A similar group was established in Asia in April 2019 to counter rapid spread of the disease in the Asia-Pacific region, where more than 60% of the world's pig population is concentrated, and a new permanent ASF expert group for the Americas is being considered. The many-year efforts resulted in the establishment of the FAO/OIE/GF-TADs platform as a progressive mechanism to combat such transboundary disease as African swine fever.

Keywords: African swine fever, epizootics, expert group, FAO, OIE.

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МЭБ и ФАО объединяют усилия, чтобы противостоять африканской чуме свиней

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РЕЗЮМЕ

Африканская чума свиней – инфекционное заболевание домашних и диких свиней, которое в XXI в. вышло за пределы своего естественного ареала (Африканский континент) и с 2007 г. (после появления в Грузии) распространилось по многим странам Европейского и Азиатско-Тихоокеанского регионов. К началу 2021 г., по данным срочных сообщений и последующих отчетов, 68% всех зарегистрированных в мире вспышек инфекции приходились на Европу. Однако наибольшие потери свиноводству нанесла вспышка, зарегистрированная в Азии в 2020 г., когда погибло 6 733 791 животное, что составляет 82% от общих мировых потерь отрасли из-за АЧС. После нескольких лет течения современной эпизоотии АЧС, при отсутствии вакцины и средств лечения, стало ясно, что серьезных проблем для свиноводческой отрасли (и в особенности мелких фермеров), а также дестабилизации мирового рынка свиноводческой продукции не избежать. В связи с этим в сентябре 2014 г. в Берне под эгидой ФАО/МЭБ GF-TADs была создана региональная Постоянная группа экспертов по АЧС в Европе (SGE ASF). Целью ее работы является налаживание более тесного сотрудничества между странами, затронутыми заболеванием, повышение транспарентности и обмен опытом профилактики и борьбы. Работа миссий постоянных экспертов по АЧС под эгидой GF-TADs в Европе показала свою эффективность и стала образцом для других регионов. В апреле 2019 г. аналогичная группа была создана и в Азии, чтобы противостоять быстрому развитию болезни в Азиатско-Тихоокеанском регионе, где сосредоточено более 60% мирового поголовья свиней. Прораба-

тывается вопрос создания новой постоянной группы экспертов по АЧС для стран Северной и Южной Америки. Результатом многолетней работы стало создание платформы ФАО/МЭБ/GF-TADs в качестве прогрессивного средства борьбы с такой трансграничной болезнью, как африканская чума свиней.

Ключевые слова: Африканская чума свиней, эпизоотия, группа экспертов, ФАО, МЭБ.

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After FMD epidemic in Europe, South America, Africa and Asia in 2001, the international community has taken concerted measures to combat transboundary infectious animal diseases, which have a significant impact on food security, public health and international trade in animal products. As a result of this work, the Global Framework for the Progressive Control of Transboundary Animal Diseases (GF-TADs) was developed and adopted, the general agreement on which was signed between the Food and Agriculture Organization of the United Nations (FAO) and the World Organization for Animal Health (OIE) on May 24, 2004. This agreement suggests the participation of the World Health Organization (WHO) in the prevention of zoonoses [1].

Taking into account the challenges that African swine fever (ASF) has brought to the countries of Europe and the Asia-Pacific region in the XXI century (Fig. 1), a regional Standing group of experts on ASF (SGE ASF) was established under the auspices of the FAO/OIE GF-TADs in Bern in September 2014 to improve cooperation between the countries affected by this disease, increase transparency and share experience in the control [2].

At the first meeting of the SGE ASF, it was decided to:

1. Establish a Standing Group of Experts on ASF.

2. Agree on the working procedure of the Standing Group of Experts on ASF. From the Russian Federation, the group of standing experts in Europe included Candidate of Science (Veterinary Medicine) N. V. Lebedev (Rosselkhoznadzor, 2014), Doctor of Science (Biology) K. N. Gruzdev and since 2017 Candidate of Science (Veterinary Medicine) A. S. Igolkin (FGBI "ARRIAH").

In its activities, the Permanent Group of Experts on ASF in Europe under the auspices of GF-TADs conducted a huge analysis study of the competent authorities' experience in the prevention and eradication of ASF outbreaks in Eastern Europe, including Ukraine, Belarus, the Baltic states, as well as in Russia, and the study of the causes of infection outbreaks. For this purpose, the experts of the GF-TADs group visited ASF infected countries as part of their missions. The data obtained were analyzed and presented at GF-TADs expert meetings: in Lithuania – in March 2015; in Belarus – in April 2015; in Latvia – in May 2015; in Russia – in May 2015; in Ukraine – in September 2015; in Estonia – in October 2015; in Moldova – in October 2016; in the Czech Republic – in October 2017; in Romania – in December 2017; in Bulgaria – in January 2019; in Belgium – in June 2019. In addition, 15 Permanent Expert Group Meetings were held in Europe (SGE ASF1–15). The meetings were attended by numerous delegates from observer countries at the invitation of the GF-TADs Chairman, Dr. B. Van Goethem.

At the 4th meeting (SGE ASF4) in May 2016 in Paris (France), during the 84th OIE General Session, new developments in the field of ASF epidemiology in the region were discussed in order to strengthen the measures taken to combat this disease after the SGE ASF3 meeting. In accordance with the recommendations of the SGE ASF3, Moldova and Romania joined the initiatives of the participating countries.

At the meeting held in May 2017, an appeal was made for increased vigilance in Romania, Hungary, Slovenia and the Czech Republic, neighboring ASF infected countries in the region. The need to share ASF awareness-raising data available in countries targeted for the general public (including carriers and tourists, hunters and farmers) was also highlighted. The collected data are published in the GF-TADs depository on African swine fever (http://web.oie.int/RR-Europe/eng/Regprog/en_ASF_depository.htm). This resource is freely available to users and is regularly updated with actual information.

The SGE ASF10 meeting was held on May 22, 2018 in Paris (France) as part of the 86th OIE General Session and was dedicated to updating of information on the epidemic situation and control measures in the SGE ASF group countries, adopted in March 2018 at the SGE ASF9 meeting. Hungary was included in the SGE ASF list of countries after confirming ASF in a wild boar found dead on April 19, 2018 on its territory.

At the SGE ASF11 meeting in 2018 in Warsaw, Poland, recommendations were approved concerning, *inter alia*, the proper management of wild boars in infected or not yet infected areas. During the event, GF-TADs experts discussed the data obtained on the ecology and epidemiology of wild boar in Europe, the experience of hunters, as well as new data on the relationship between ASF outbreaks in the population of wild boar and domestic pigs in infected areas.

As a result, the meeting emphasized the need for the early release of the "OIE Handbook on African Swine Fever in Wild Boars and Biosecurity during Hunting" (V. Guberti et al.), with the possibility of updating the document as necessary.

The SGE ASF12 meeting was held on March 11 and 12, 2019 in Prague (Czech Republic) in conjunction with the BTSF (Better Training for Safer Food) meeting. The event included a technical training on ASF eradication based on the experience of the Czech Republic in eliminating this infection in the wild boar population.

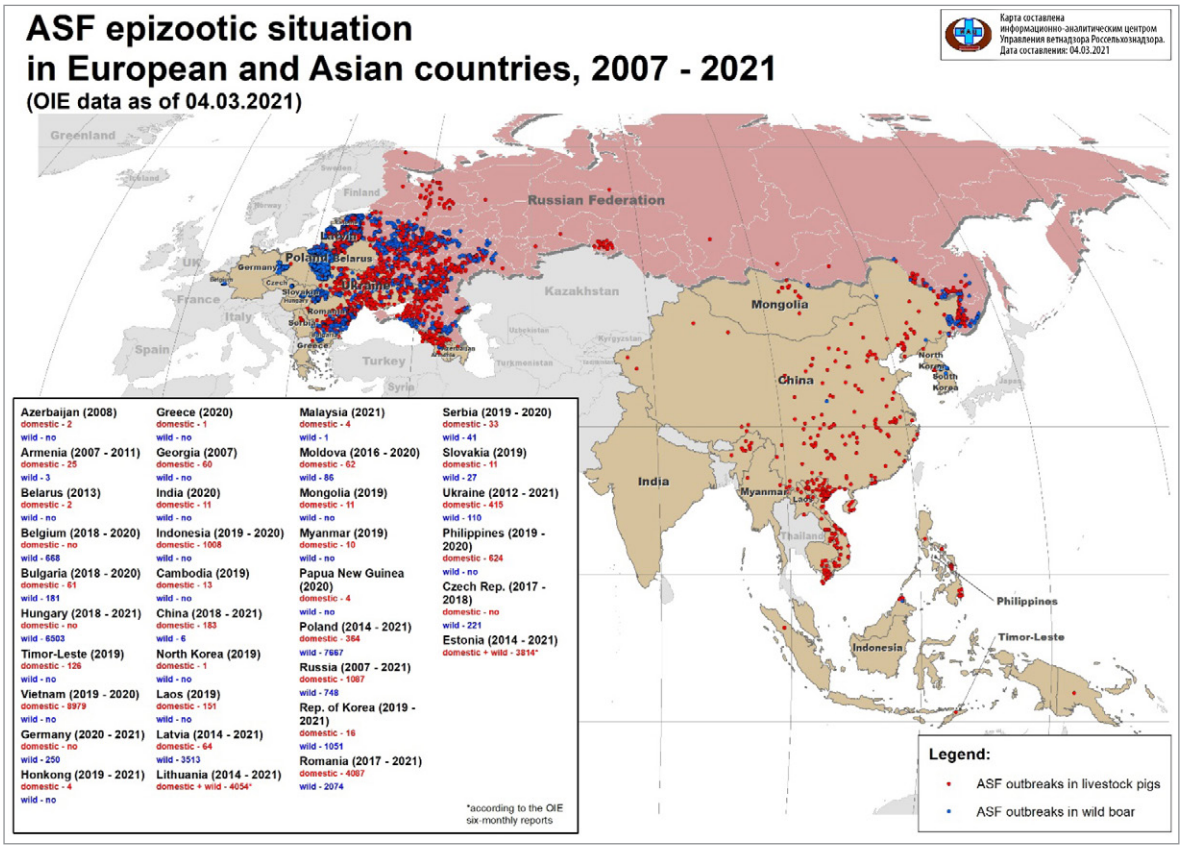

Fig. 1. ASF epidemic situation in the Russian Federation, European and Asian countries, 2007–2021 (the map is prepared by the officers of the Information Analysis Centre, FGBI "ARRIAH")

Рис. 1. Эпизоотическая ситуация по АЧС в Российской Федерации, странах Европы и Азии, 2007–2021 гг. (данная карта подготовлена сотрудниками информационно-аналитического центра ФГБУ «ВНИИЗЖ»)

The SGE ASF13 meeting was held on May 29, 2019 in Paris (France) as a side event of the OIE 87th General Session, attended by representatives of ASF-infected European countries, as well as observers from around the world. Thirteen ASF infected European countries reported on the current situation and the measures taken. The OIE Regional Representation in Asia provided information on the disease situation in its region and the steps taken to establish a working group of experts on ASF in the region [3], and the delegate from Canada reported on recent initiatives being implemented in the Americas to improve ASF prevention and preparedness.

The extended meeting of SGE ASF14 was held in September 2019 in Sofia (Bulgaria) under the chair of the OIE Director General, Dr. Monique Eloit, during which the Balkan countries joined the Standing Group of Experts on ASF in Europe (SGE ASF GF-TADs) [4].

The participants of the meeting pointed out that the consequences of the current global ASF crisis are a serious problem for the pig industry; they call into question the possibility of small farming existence in future and destabilize the global market of pig products.

Taking into account the analysis of the data collected so far during the GF-TADs expert missions in Europe, two main patterns of ASF epidemic are observed:

1) in most countries, the disease occurs mainly in wild boar populations, sometimes with zero outbreaks in domestic pigs;

2) the disease in the population of domestic pigs occurs mainly in small-scale farms and backyard farms.

But the possibility of ASF outbreaks at a significant distance from the infected areas should not be excluded (for example, in the Czech Republic in June 2017 the outbreak occurred more than 500 km from the infected areas of Poland and Ukraine; in Belgium in August 2018 – about 1,000 km from the nearest infected area in Poland) [5].

The experience gained by the international community has once again shown that in order to stop the spread of this disease, with no vaccines available, effective coordination of actions, cooperation and exchange of knowledge between all stakeholders, both at the international (national) and regional levels, is necessary. ASF Control is possible only if all veterinary regulations are strictly complied with [4].

The fifteenth meeting of the Standing Group of Experts on ASF (SGE ASF15), originally scheduled to be held face-to-face in Slovakia, due to the new coronavirus infection (COVID-19) unfavorable situation in the world, was held on May 6, 2020 in the form of a teleconference. It was attended by representatives of 31 European countries, as well as China and Japan. The speakers from the SGE ASF member countries in Europe briefly described the national ASF situation, focusing on the changes that have occurred since the last meeting in Sofia in September 2019.

The positive experience of anti-crisis management of the ASF situation in Belgium was noted.

Report on the results of the SGE ASF expert mission to Serbia conducted in December 2019 (with the participation of Doctor of Science (Biology) K. N. Gruzdev) was

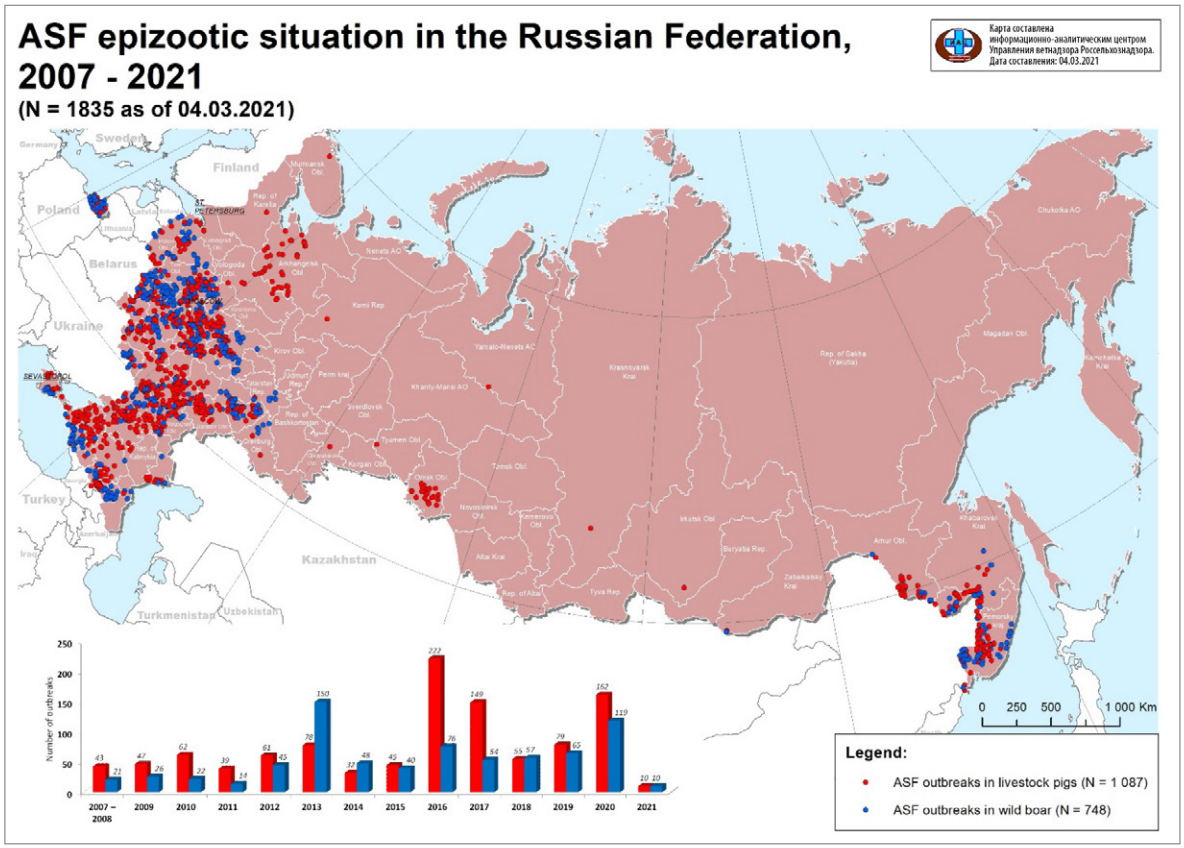

Fig. 2. ASF epidemic situation in the Russian Federation, 2007–2021 (the map is prepared by the officers of the Information Analysis Centre, FGBI "ARRIAH")

Рис. 2. Эпизоотическая ситуация по АЧС в Российской Федерации, 2007–2021 гг. (данная карта подготовлена сотрудниками информационно-аналитического центра ФГБУ «ВНИИЗЖ»)

approved without comments and posted on the GF-TADs page of the OIE website for Europe.

Scheduled for April 2020 mission of SGE ASF experts to Slovakia was postponed due to the COVID-19 pandemic.

In 2020 the OIE and FAO, in collaboration with GF-TADs, have developed the GF-TADs initiative for the Global control of ASF online platform, which summarizes the experience of ASF eradication in modern conditions and contains a strategy for progressive control of the disease [6, 7]. At the end of July 2020, this platform was launched [8]. It is expected that FAO/OIE, together with GF-TADs, will develop this initiative by supporting national, regional and global partnerships, improving prevention and preparedness measures, and minimizing the adverse effects of ASF [9].

The Russian Federation cooperates extensively with the FAO and the OIE, initiating numerous proposals for the prevention and control of infectious animal diseases, including ASF. Representatives of our country actively participate in the work of various groups, commissions of these organizations, meetings, symposiums, conferences, as well as the OIE General Sessions. Since the introduction of ASF in Georgia in 2007, the Rosselkhoznadzor urges the world veterinary community and the veterinary services of neighboring countries to actively cooperate and share their experience in the prevention and control of ASF. The reports of the Russian Federation on the detection of infectious diseases sent to the OIE through the World Animal Health Information System (WAHIS) are transparent and fully reflect the epizootic situation in the country (Fig. 2).

The Rosselkhoznadzor is actively working to strengthen national sanitary measures, including biosafety in pig farms, measures to regulate the number of wild boar, conducts educational work, updates regulations, organizes the exchange of scientific information on the etiology, epizootology, and the use of safe methods in the entire chain of production and sale of pig products.

CONCLUSION

In the XXI century, African swine fever (of domestic and wild pigs) has expanded its habitat. In recent years, the number of countries and territories in Europe and Asia affected by ASF has increased. Recognizing the increased risk of this disease in any country in the world and its significant impact on the development of pig farming, trade, food security, national and global economies, FAO and the OIE are taking active measures to counter ASF. The FAO/OIE GF-TADs platform proves itself as a progressive mechanism for combating such a cross-boundary disease as ASF.

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OIE REFERENCE LABORATORY FOR HIGHLY PATHOGENIC AVIAN INFLUENZA AND LOW PATHOGENIC AVIAN INFLUENZA (POULTRY) AND NEWCASTLE DISEASE
РЕФЕРЕНТНАЯ ЛАБОРАТОРИЯ МЭБ ПО ВЫСОКОПАТОГЕННОМУ И НИЗКОПАТОГЕННОМУ ГРИППУ ПТИЦ И НЬЮКАСЛСКОЙ БОЛЕЗНИ

Announcement:

A study guide has been published by the FGBI "ARRIAH"

Sheep pox and goat pox: a study guide / A. V. Kononov, O. P. Byadovskaya, K. A. Shalina, A. V. Sprygin, V. I. Diev. –
Vladimir: FGBI "ARRIAH", 2021. – 46 p.: ill.
ISBN 978-5-900026-75-6

The study guide is devoted to the general aspects and features of the epidemic process, diagnosis and prevention of sheep pox and goat pox. The discussed diseases are widespread almost all over the world, including the territory of the Russian Federation. They cause significant economic damage.

The guide describes in detail the manifestations of the disease (pathogenesis, clinical signs, post mortem lesions), modern aspects of diagnosis, prevention and control measures.

The study guide includes original illustrations obtained by the authors during sheep pox research activities.

The textbook is intended for students, trained in the veterinary field (students, postgraduates, students of advanced training courses) as a source of modern knowledge on important infectious diseases, - sheep pox and goat pox. The presented content can be useful for livestock industry specialists and epizootologists, as well as practicing veterinarians.

Announcement:

Training Courses in the FGBI "ARRIAH" in May 2021

In May 2021, a number of training courses are to be held in the FGBI "ARRIAH" for veterinarians from the Rosselkhoznadzor Territorial Administrations, from the Veterinary Departments of Republics, Krais and Oblasts and veterinary laboratories of the Subjects of the Russian Federation and the CIS countries. The training are:

– **May 12, 2021** – a webinar training will be carried out in **"Epizootological peculiarities of notifiable avian mycoplasmoses (*Mycoplasma gallisepticum*, *Mycoplasma synoviae*), principles of their eradication in commercial poultry farming, prevention and treatment in the Russian Federation"**.

The webinar will last for 4 academic hours. The webinar cost per 1 student is 1,320 rubles, including VAT. Information on spread of avian mycoplasmoses in the world and in the Russian Federation will be provided at the webinar; such topics as epizootology and diagnosis of mycoplasmoses etc. will be addressed;

– **from May 17 to 21, 2021**, a webinar training will be carried out within the program of additional professional education in **"Current zoonoses"**.

The webinar will last for 24 academic hours. The webinar cost per 1 student is 5,820 rubles, including VAT. Topics to be addressed during the webinar are related to such infectious diseases as anthrax, brucellosis, animal tuberculosis, campylobacteriosis, bovine spongiform encephalopathy and other prion diseases of animals, glanders, dermatophytoses, etc.;

– **from May 24 to 28, 2021**, a training will be carried out within the program of additional professional education in **"Epizootology, diagnosis, prevention and control measures for foot-and-mouth disease, sheep pox and goat pox, peste des petis ruminants under modern conditions"**.

The training will last for 72 academic hours, both on-site and off-site. The training cost per 1 student is 85,000 rubles, including VAT. Topics to be addressed during the training are related to such infectious diseases as foot-and-mouth disease, sheep and goat pox, peste des petits ruminants, the training also includes clinical examination of sick animals, post-mortem examination and sampling for laboratory tests.

Educational activities are performed pursuant to License No. 001671 dated 03.08.2011 (Registration Number 1603) issued by the Federal Service for the Supervision of Education and Science.

Leading researchers of the FGBI "ARRIAH" conduct the trainings. Upon completion, a certificate of participation in the webinar or a certificate of professional development is issued.

Detailed information on enrolment procedure is available on the website of the FGBI "ARRIAH" – http://www.arriah.ru in section "Participation in training webinars and seminars".

Applications for the webinar should be sent to: *mail@arriah.ru*.

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