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Detection of antibodies to non-structural proteins of foot-and-mouth disease virus (review)

A. S. Yakovleva, A. V. Scherbakov
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SUMMARY

Foot-and-mouth disease (FMD) is a highly contagious viral disease of cloven-hooved animals that can cause epidemics and great economic losses. The disease remains a huge problem in developing countries and poses a constant threat to developed countries. Detection of antibodies to FMD virus non-structural proteins in the blood of susceptible animals is an important tool for the disease control. This is the only way for reliable differentiation of vaccinated animals from convalescent and virus-carrier animals. Various modifications of solid-phase enzyme immunosorbent assay (ELISA) have been developed for detection of antibodies to FMD virus nonstructural proteins. Recombinant FMD virus non-structural proteins, mostly 3ABC or 3AB, are used as an antigen for such assay. In a short time, recombinant FMD virus antigen-based ELISA has evolved from an in-house laboratory method to commonly available commercial test systems, most of which have high diagnostic specificity and sensitivity. The said method is widely used for FMD surveillance. In the countries and zones free from FMD without vaccination the ELISA for detection of antibodies against FMD virus non-structural proteins is used as a primary method for FMD serological monitoring and retrospective diagnosis. In the countries and zones free from FMD with vaccination this ELISA is used for confirmation of the virus infection absence in vaccinated herds. In South America, ELISA for detection of antibodies against FMD virus non-structural proteins was used for detection of infected animals during the disease eradication. Currently, it is used for monitoring for the virus circulation in still FMD infected Asian and African countries implementing progressive FMD control programme. In Russia having zones where anti-FMD vaccination is carried out this method is a mandatory tool of the disease surveillance. The review is based on the analysis of 65 publications.

Keywords: review, foot-and-mouth disease virus, non-structural proteins, enzyme-linked immunosorbent assay

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Обнаружение антител к неструктурным белкам вируса ящура (обзор)

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РЕЗЮМЕ

Ящур – высококонтагиозное вирусное заболевание парнокопытных животных, способное вызывать эпизоотии и наносить большой экономический ущерб. Болезнь остается огромной проблемой в развивающихся странах и представляет постоянную угрозу для развитых стран. Важным инструментом контроля заболевания является обнаружение антител к неструктурным белкам вируса ящура в крови восприимчивых животных. Это единственный способ, позволяющий достоверно дифференцировать вакцинированных животных от переболевших и вирусоносителей. Для выявления антител к неструктурным протеинам вируса ящура разработаны различные модификации твердофазного иммуноферментного анализа. В качестве антигена в них используются рекомбинантные неструктурные белки вируса ящура, чаще всего 3ABC или 3AB. За короткий срок иммуноферментный анализ с использованием рекомбинантных антигенов вируса ящура прошел путь от внутрилабораторного метода до общедоступных коммерческих тест-систем, большинство из которых обладают высокой диагностической специфичностью и чувствительностью. Данный метод широко применяется в надзоре за ящуром. В странах или зонах, благополучных по ящуру без вакцинации, иммуноферментный анализ, основанный на обнаружении антител к неструктурным белкам вируса ящура, применяется как основной метод серологического мониторинга и ретроспективной диагностики заболевания. В благополучных по ящуру странах и зонах с вакцинацией этот метод используется для доказательства отсутствия вирусной инфекции в вакцинированных стадах. В Южной Америке при эрадикации заболевания иммуноферментный анализ, основанный на обнаружении антител к неструктурным белкам вируса ящура, применялся для выявления инфицированных животных, а в настоящее время этот метод используется для мониторинга циркуляции возбудителя в еще неблагополучных по заболеванию странах Азии и Африки, реализующих программу прогрессивного контроля за ящуром. В России, как стране с зонами вакцинации против ящура, данный метод является обязательным инструментом надзора за заболеванием. Обзор составлен на основе анализа 65 источников.

Ключевые слова: обзор, вирус ящура, неструктурные белки, иммуноферментный анализ

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INTRODUCTION

Foot-and-mouth disease (FMD) is a highly contagious disease of domestic and wild cloven-hooved animals manifested by vesicular lesions in oral epithelium, limbs and udder [1]. The disease belongs to transboundary diseases capable of crossing the borders between the countries and causing huge economic losses in animal farming industry.

The disease agent is non-enveloped RNA virus of foot-and-mouth disease virus belonging to *Aphthovirus* genus of *Picornaviridae* family [2]. There are seven FMD virus serotypes*: O*, A, C, Asia-1, SAT-1, SAT-2 and SAT-3. Each FMDV serotype is classified into topotypes, genetic lines and sublines [3, 4].

FMD is controlled by preventive vaccination of susceptible livestock animals in enzootic areas and in buffer zones established in some disease-free countries (for example, in Russia) along the borders to the disease-affected countries. Immunization prevents the clinical disease however not always is capable of preventing subclinical infection and virus persistence [5]. Vaccinated animals that have been FMDV infected could potentially become the infection source for other susceptible animals [6]. Therefore, FMD surveillance programme in the countries and zones where vaccination is practiced envisages obligatory proving the virus infection absence in vaccinated herds. Antibodies to FMDV non-structural proteins is the most reliable indicator of the infection in vaccinated animals [7–10].

Four structural proteins (1A, 1B, 1C and 1D) and ten non-structural proteins (L, 2A, 2B, 2C, 3A, $3B_{1-3}$, 3C and 3D) are synthesized in equal amounts during FMDV replication in cells (Fig. 1). Non-structural proteins perform various functions during the virus replication in infected cells: polymerase, helicase, protease functions and etc. Functions of some FMDV non-structural proteins remain unclear [1, 11]. In contrast to structural proteins, non-structural proteins are highly conserved, i.e. they do not differ in different FMDV serotypes when tested with serological methods.

The virus-infected animals develop antibodies to all viral proteins: both to structural and non-structural proteins. Anti-FMD vaccinated animals have a distinct antibody profile: only antibodies against the viral structural proteins are, as a rule, detected in such animals. This is accounted for the fact that modern anti-FMD vaccines are subjected to purification and most of the viral non-structural proteins together with cellular debris are removed from the vaccines during this stage [12, 13]. Thus, detection of FMDV non-structural proteins can serve as evidence that the animal is infected with the virus, regardless of whether it has been vaccinated or not.

DEVELOPMENT OF SEROLOGICAL METHODS FOR DETECTION OF ANTIBODIES AGAINST FMDV NON-STRUCTURAL PROTEINS

Different techniques for detection of antibodies against FMDV non-structural proteins were tested: agar gel immunodiffusion assay [14], immunoblotting [15], etc. However, solid-phase immunosorbent assay (ELISA) has turned out to be the most technologically-advanced method [16].

Development of the ELISA intended for detection of antibodies to FMDV non-structural proteins (NSP-ELISA) requires addressing the antigen preparation challenge. Since preparation of purified FMDV non-structural proteins from infected cell culture is difficult, all researchers used chemically synthesized peptides [17, 18] or, much more often, recombinant proteins [19] as an antigen in NSP-ELISA test-kits. Recombinant proteins were generated by molecular cloning and expression of relevant FMDV genes in *Escherichia coli* (*E. coli*) [19–23] or in baculovirus-insect cells system [24–27].

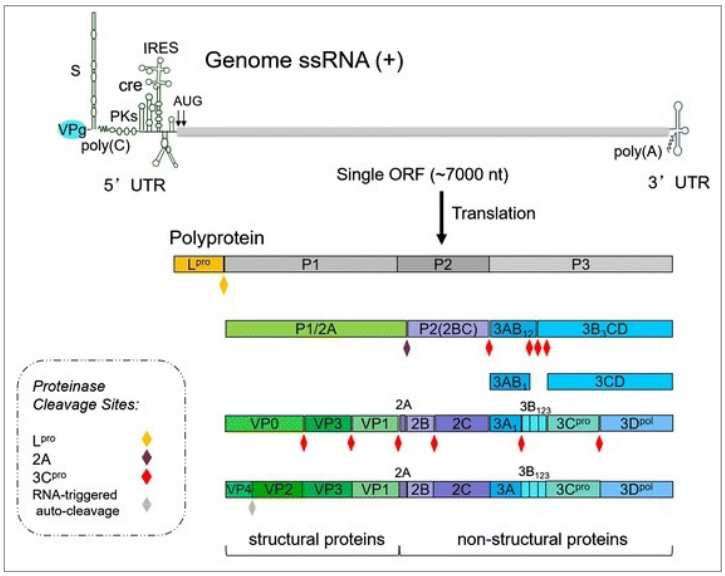

Fig. 1. Schematic diagram of FMD virus genome and processing of viral proteins [11]

Almost all non-structural proteins of FMDV have been tested as antigens for NSP-ELISA test-kit [28–32]. Multiple tests have showed that NSP-ELISA test-kit based on 3AB or 3ABC recombinant proteins provides the best differentiation between post-infection and postvaccinal antibodies [8, 20, 21, 23, 32–37]. Antigenicity of 3 ABC polyprotein appears to be determined by 3A and 3B proteins being its parts, role of 3C protein is significantly lower. Thus, it is shown that 3C protein does not to react with hyperimmune serum when tested by immunoblotting and so does not contain linear epitopes recognized by antibodies [34].

Several solid-phase ELISA variants were developed for detection of antibodies to FMDV non-structural proteins. Anti-species (i.e. species-specific) conjugate was used for indirect ELISA for detection of antibodies bound to FMDV non-structural antigen. Therewith, indirect ELISA in most cases allows for testing sera from cattle, small ruminants as well as from pigs. Enzyme-labeled polyclonal [38] or monoclonal [24] antibodies against FMDV non-structural proteins were used as detection antibodies for competitive ELISA. This ELISA variant allows for testing of sera from animals of all species. In 2015, M. Hosamani et al. proposed to use horseradish peroxidase-conjugated G protein for indirect 3ABC ELISA. This allowed for testing of sera from animals of different species with indirect ELISA [39].

Most described ELISA test-systems based on 3ABC protein or its components demonstrated high specificity and sensitivity. NSP-ELISA allowed detection of post-infection antibodies to FMDV within 12–18 months after infection starting with day 7–10 after infection [21, 23, 28, 40–42]. E. Elnekave et al. [43] have managed to detect antibodies 3 years after infection and W. B. Chung et al. have managed to detect antibodies 3.5 years after infection [25].

Initially, NSP-ELISA was used only in the developer laboratories, then it was commercialized. Currently, several companies (Bionote, IDEXX Laboratories, Inc., ThermoFisher Scientific, Inc. (USA), IDVet (France), IZSLER (Italy), etc.) produce commercial test-kits for detection of antibodies against FMDV non-structural proteins.

There are several publications aimed at comparative assessment of the commercial test-kits [42, 44–46]. E. Brocchi et al. [47] carried out the most extensive comparative study. They compared six diagnostic kits: CHEKIT-FMD-3ABC (Bommeli Diagnostics, Switzerland); UBI® FMDV NS EIA (United Biomedical, Inc., USA); I-ELISA 3ABC/EITB (PANAFTOSA, USA); Ceditest® FMDV-NS ELISA (Cedi Diagnostics B.V., Netherlands); 3ABC trapping-ELISA (IZS-Brescia, Italy); SVANOVIR™ FMDV 3ABC-Ab ELISA (Svanova, Sweden). A total of 3,551 sera from cattle and small ruminants from 9 countries were tested with each test-kit. All tested kits demonstrated high specificity (> 96%), however their sensitivity varied significantly. The following test-kits demonstrated the highest relative sensitivity: 3ABC trapping-ELISA (100%), I-ELISA 3ABC/EITB (99.6%) and Ceditest® FMDV-NS ELISA (99.6%), the sensitivity of the following test-kits was significantly lower: UBI® FMDV NS EIA (88.8%), SVANOVIR™ FMDV 3ABC-Ab ELISA (83.6%) and CHEKIT-FMD-3ABC (81.5%).

Since the diagnostic specificity of all test systems is not absolute, false positive results could be obtained when the said test-kits are used for serological diagnosis of foot-and-mouth disease. It is recommended to use confirmatory test that may be either NSP-ELISA of other manufacturer or immunoblotting, to address this problem [8, 48].

NSP-ELISA FOR FMD SURVEILLANCE

Currently, the World Organization for Animal Health (WOAH) recommends to use NSP-ELISA as an official method for serological diagnosis of FMD [48].

For countries free from FMD without vaccination NSP-ELISA is an ideal tool for FMD seromonitoring and retrospective diagnosis since it allows for detection of antibodies to all seven FMDV serotypes using single test [8, 49].

Detection of antibodies to FMDV non-structural proteins is a key diagnostic tool for FMD surveillance in the regions where this disease is enzootic [50]. NSP-ELISA was used as a screening method for identification of infected animals in vaccinated herds during FMD eradication campaign in South America. FMD-infected countries that have adopted and implement national roadmaps on progressive FMD control in the framework of the Progressive Control Pathway for Food-and-Mouth Disease developed by the Food and Agriculture Organization of the United Nations (FAO) use NSP-ELISA for the virus circulation monitoring [1].

Countries applying for FMD-free status or recovery of the lost FMD-free status should submit a dossier to the WOAH, which, among other things, should contain the results of testing of animals for antibodies to FMDV non-structural proteins. Countries after being recognized as FMD-free should continue serological testing for the free status confirmation.

NSP-ELISA is a mandatory tool used to control transboundary transportation of live animals and animal products. International regulations governing the trade in livestock animals require testing of horned livestock and pigs imported from countries or zones with vaccination for antibodies to the FMDV [51].

The wide use of NSP-ELISA for diagnostic and monitoring tests for foot-and-mouth disease has revealed multiple advantages of the method as well as certain limitations related to its use. The advantages include high diagnostic sensitivity and specificity, throughput capacity, the ability to detect antibodies to all FMDV serotypes using single test.

The limitations related to NSP-ELISA use are accounted for not so much the method characteristics as the peculiarities of the tested samples. Early studies have already shown that NSP-ELISA detects antibodies in some vaccinated animals [7, 52–56]. S. P. Chen et al. using PrioCHECK FMDV NS test-kit (ThermoFisher Scientific, Inc., USA) found that non-vaccinated or once vaccinated pigs were seronegative, but antibodies to non-FMD virus structural proteins were detected in 16.2% of animals vaccinated several times [54]. The study performed by G. K. Sharma et al. showed that the specificity of NSP-ELISA used for tests of non-vaccinated animals was very high and reached 100% [42]. However, antibodies to FMDV non-structural proteins were detected in 33% of animals when samples were collected from animals on day 14 after vaccination and tested with PrioCHECK FMDV NS. Such large number of animals having antibodies to FMDV non-structural proteins can be accounted for the fact that the Indian vaccine was not appropriately purified from the virus non-structural proteins (i.e. not compliant with the requirements for the vaccine purity). However, there are

Fig. 2. FMD-NSP-ELISA test-kit for detection of antibodies against FMD virus non-structural proteins produced by the FGBI "ARRIAH"

other multiple data evidencing that some animals develop antibodies against FMDV non-structural proteins after their vaccination. Thus, in experiments carried out by T. Tekleghiorghis et al. seven out of one hundred cattle had antibodies to FMDV non-structural proteins after single vaccination [57]. It should be noted that the level of such antibodies was low: the maximum percent inhibition was 58% at cut-off of 50% when the sera were tested with PrioCHECK FMDV NS test-kit.

In studies conducted by Chinese experts, the number of vaccinated cattle with antibodies against the FMDV non-structural proteins correlated with the number of vaccinations: there were 2.15% of seropositive animals among the animals vaccinated up to 10 times and already 5.93% of seropositive animals among the animals vaccinated up to 15 times [58].

Vaccine purification appears not to completely remove FMDV non-structural proteins from the vaccine [58, 59]. This was obviously the circumstance that forced the WOAH to amend requirements for anti-FMD vaccines. According to previous rules, the vaccine shall not induce antibodies to FMDV non-structural antibodies after three vaccinations and according current WOAH Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, it is allowed that one of eight animals vaccinated twice has such antibodies [48].

Thus, possible detection of seropositive animals having postvaccinal but not post-infection antibodies to FMDV non-structural proteins should be taken into account when NSP-ELISA is used for diagnostic and monitoring tests carried out in zones where vaccination is practiced.

USE OF NSP-ELISA IN RUSSIA

In the Russian Federations studies aimed at development of ELISA for detection of the antibodies to FMDV non-structural proteins were carried out at the Federal Centre for Animal Health (FGBI "ARRIAH", Vladimir). Recombinant FMDV proteins 3A, 3B and 3AB were produced by expression in *E. coli* [60]. Indirect ELISA for detection of antibodies to FMDV non-structural proteins in cattle sera was developed based on the recombinant antigens [61, 62]. Later, improved 3AB-ELISA variant allowing testing not only cattle sera but also sera from small ruminants was proposed [63]. This method passed validation showing that its diagnostic specificity and sensitivity was 99.8% and 96.6%, respectively [64]. This method is included in the scope of the FGBI "ARRIAH" accreditation.

Since Russia is one of the countries that have zones where anti-FMD vaccination is carried out, NSP-ELISA is a mandatory tool for serological monitoring of the disease. During the serological surveillance the algorithm for diagnostic testing of sera recommended by the WOAH for FMD [51] as well as used in medicine for diagnosis of some highly significant diseases, for example AIDS, is used[1]. Its essence lies in the use of two test-systems: screening and confirmatory. In the FGBI "ARRIAH" initial screening of samples is carried out with 3AB-ELISA developed by A. S. Yakovleva et al. [64], and then all positive samples are tested again with commercial PrioCHECK FMDV NS test-kit. The sample is considered positive when it is tested positive with both tests-systems. Thus, usage of screening and confirmatory tests allows for exclusion of false positive results during diagnostic and monitoring tests [65].

About 250,000 serum samples from cattle and small ruminants submitted from all Russian Federation regions were tested with NSP-ELISA at the FGBI "ARRIAH" within the federal FMD monitoring in 2015–2022. Serological test results were included as evidence in the dossiers

[1] SanPiN 3.3686–21 Sanitary and epidemiological requirements for infectious disease prevention approved by the Ordinance of the Chief Medical Officer of the Russian Federation No. 4 of 28 January 2021. Available at: https://docs.cntd.ru/document/573660140.

submitted to the WOAH. The WOAH based the said dossiers granted the status of the country with FMD free zone without vaccination (the zone includes 52 Subjects of the Russian Federation) and status of the country with three FMD free zones with vaccination (the zones include another 16 Subjects of the Russian Federation) to the Russian Federation[2].

The FGBI "ARRIAH" produces commercial FMD NSP-ELISA test-kit for detection antibodies to FMDV non-structural proteins to provide regional veterinary laboratories of the Russian Federation with up-to-date tools for FMD serological diagnosis (Fig. 2)[3].

CONCLUSION

Detection of antibodies to FMDV non-structural proteins is an important tool for the disease surveillance. Ability of FMDV NSP-tests to detect the infection regardless of the virus serotype has determined their demand in the countries that are FMD free without vaccination and ability of NPS-ELISA tests to detect the infection in vaccinated animals results in their wide use in the countries or zones where vaccination is practiced. However, it should be noted that differentiation between vaccinated and infected animals with FMD NSP-tests becomes possible only if highly purified anti-FMD vaccines are used.

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Current approaches to development of real-time qPCR test-kits

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SUMMARY

Currently fluorescent quantitative real-time polymerase chain reaction, which is a cutting-edge technology in genetic diagnosis, is used in different areas of molecular biology. Practical advantage of simplicity as well as combination of high speed, sensitivity and specificity made it possible to use this analysis for nucleic acid quantitation. The paper presents general information and recommended rules for the development of real-time qPCR. The publication is aimed to acquaint the researchers and reviewers with necessary requirements to be followed in order to ensure high accuracy, reliability and transparency of the experiments, correct interpretation and repeatability of the test results. Current approaches are described that allow obtaining reliable and consistent results by different operators, at different times and in different laboratories. Basic requirements for reagents used, nucleotide sequences and validation methods are given. In general, the publication gives the information needed to achieve three ultimate goals: to provide the authors with a broad range of tools and requirements for the development of real-time qPCR based-techniques; to give the possibility to the reviewers and editors of assessing the quality of articles and guidelines/instructions in accordance with the required criteria; to obtain consistent and reliable results of tests performed using this method.

Keywords: review, real-time polymerase chain reaction, reverse transcription, requirements for test-kits, validation, oligonucleotide primers and probes, performance of amplification reaction

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Современные подходы к разработке тест-систем на основе количественной ПЦР в режиме реального времени

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РЕЗЮМЕ

В настоящее время в различных областях молекулярной биологии применяется основанная на флуоресцентной детекции количественная полимеразная цепная реакция в режиме реального времени, которая является передовой технологией генной диагностики. Практическая простота, а также сочетание высокой скорости, чувствительности и специфичности сделали возможным использование данного анализа для количественного определения нуклеиновых кислот. В статье представлены общие сведения и отражены рекомендуемые правила для разработки методик количественного ПЦР-анализа в режиме реального времени. Материалы публикации нацелены на предоставление исследователям и рецензентам необходимых требований, которых следует придерживаться, чтобы обеспечить высокую точность, надежность и прозрачность экспериментов, правильную интерпретацию и повторяемость результатов анализа. Представлены современные подходы, которые позволяют получать надежные и достоверные результаты, проводимые разными операторами, в разное время и в разных лабораториях. Приведены основные требования, предъявляемые к применяемым реагентам, перечням нуклеотидных последовательностей и методам проведения валидационного анализа. В целом в представленной публикации отражена информация для достижения трех конечных целей работы: предоставить авторам широкий арсенал инструментов и требований для разработки методик на основе количественной полимеразной цепной реакции в режиме реального времени; дать возможность рецензентам и редакторам оценивать качество представленных материалов статей и методических рекомендаций/указаний в соответствии с требуемыми критериями; получать однородные, сопоставимые и надежные результаты исследований, выполненных с помощью данного метода.

Ключевые слова: обзор, полимеразная цепная реакция в режиме реального времени, обратная транскрипция, требования к тест-системам, валидация, олигонуклеотидные праймеры и зонды, эффективность реакции амплификации

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INTRODUCTION

The fluorescence-based quantitative real-time PCR (qPCR) is a cutting-edge technology in genetic diagnosis used in different areas of molecular biology [1–3]. Its practical simplicity, together with its combination of speed, sensitivity, and specificity have made it useful for nucleic acid quantitation [4–6]. In recent years, many qPCR-based diagnostic applications have been developed, including microbial quantification, gene dosage determination, identification of transgenes in genetically modified foods, risk assessment of cancer recurrence, etc. [4–15].

Currently, there is a lack of consensus on how best to perform qPCR experiment development followed by data analysis. The problem is exacerbated by the lack of precise requirements for these developments to permit the investigator to critically evaluate the quality of the results presented [16–18]. Thus, some authors make a number of technical errors while developing qPCR-based test-kits. Some of them are presented in Table 1.

Consequently, there is the real danger of inadequate and conflicting results obtained by qPCR [19, 20]. In addition, information about sample acquisition and handling, RNA quality and integrity, reverse transcription details, PCR efficiencies, and analysis parameters is frequently omitted, whereas sample normalization is carried out against single reference genes without adequate justification [21]. The qPCR method standardization problem has been reported in many scientific publications [19–23], therefore, this task is currently relevant.

Table 1
Possible technical errors that affect real-time qPCR assay performance and their negative consequences

No.	Possible technical errors	Negative consequences
1	Inadequate sample storage, preparation, and nucleic acid quality	Yielding highly variable results
2	Poor choice of oligonucleotide primers and probes for the PCR	Inefficient and less-than-robust assay performance;
3	Inappropriate statistical analyses	The results obtained may be misleading

There are a number of publications, in particular guidelines for qPCR method development, which reflect the basic requirements for the development of such techniques [24–26].

Applications of qPCR technology are currently used for research and diagnostic purposes [23]. Research applications usually analyze a wide range of targets with a fairly low throughput and many different sample types [25]. The main parameters that need to be addressed relate to assay analytical sensitivity and specificity [20, 26].

Diagnostic applications usually analyze a limited number of targets, but require high throughput protocols that are targeted at only a few sample types [22]. The test characteristics include information on analytical sensitivity and specificity that in this context refers to how often the assay returns a positive result when a target is present and how often it is negative in the absence of the target [19, 24]. In addition, tests for accuracy, repeatability and reproducibility of the analysis are required (Table 2).

The aim of this document is to provide authors and reviewers general current requirements that should be followed when developing qPCR-based methods in order to ensure high accuracy and reliability, experimental transparency, correct interpretation and repeatability of the analysis results.

STANDARDIZATION OF SOME TERMS USED IN THE DEVELOPMENT OF QPCR-BASED METHODS

In accordance with the proposals available in Real-time PCR Data Markup Language (RDML) resource[1] [27], it is required to make some edits to the use of generally accepted terms used in the development of PCR-based techniques, in particular:

– TaqMan probes should be referred to as hydrolysis probes;

– the term FRET probe (fluorescence resonance energy transfer probe) refers to a generic mechanism in which emission/quenching relies on the interaction between the electron-excitation states of two fluorescent dye molecules;

– LightCycler probes should be referred dual hybridization probes [25];

[1] Real-time PCR Data Markup Language. Available at: https://rdml.org.

– the nomenclature describing the fractional PCR cycle used for quantification is inconsistent, with threshold cycle (C_t), crossing point (C_p), and take-off point (TOP) currently used in the literature. To unify the nomenclature, many authors use the term "quantification cycle" (C_q) [28, 29].

GENERAL REQUIREMENTS FOR DIFFERENT ANALYTICAL STAGES

Sampling of biological material and sample storage. Sampling can be responsible for the experimental variability due to fragile nature of RNA. According to P. Micke et al. [30], fresh biological material can be kept on ice without any major influence on RNA quality, but this approach cannot be applied everywhere. It is essential to record the entire history of sampling and transportation of biological samples in PCR protocols [31].

Nucleic acid extraction. Nucleic acid extraction is also a critical step in operations the qPCR setup [32, 33]. Extraction efficiency depends on adequate homogenization, the type of sample (e.g., tissue homogenate, culture suspension, saliva, blood, etc.), target density, physiological status (e.g., healthy, cancerous, or necrotic), genetic complexity, and the amount of biomass processed [27, 34–36].

Therefore, it necessary that details of the nucleic acid extraction method be provided and that the methods used for measuring nucleic acid concentration and assessing its quality be described. Such details are particularly important for RNA extracted from fresh frozen biological samples, because variations in tissue-preparation procedures have a substantial effect on both RNA concentration and quality [37–39].

Quality control of RNA extracts

Quantification of RNA in extracts. Quantification of RNA in the extracted samples is important for the correct analysis of nucleic acids at the stage of reverse transcription and amplification reactions.

There are several groups of RNA quantification methods, namely:

1) spectrophotometry using various spectrophotometers;

2) microfluidic analysis, for example, using bioanalyzer systems by Agilent Technologies, Inc. (USA), Bio-Rad Laboratories, Inc. (USA), etc.;

3) capillary gel electrophoresis, for example, using the QIAxcel instrument by Qiagen (Germany), etc.;

4) fluorescent dye detection (Ambion, RiboGreen, Thermo Fisher Scientific, Inc., USA, etc.) [14, 23, 40].

It is worth remembering that RNA degrades markedly *in vivo*, owing to the natural regulation of mRNAs in response to environmental factors [41, 42]. Moreover, even high-quality RNA samples can show differential degradation of individual molecules, which is difficult for a researcher to control.

RNA sample purification quality assessment. To assess the purity of the prepared samples, a spectrum analysis is performed. The sample absorbance spectrum is measured at a wavelength of 205–325 nm and a temperature of 20–22 °C. In RNA samples, the content of residual phospholipids, polysaccharides and guanidine isothiocyanate, carbolic acid, polypeptides and suspended particulate matter is evaluated by determining the optical density values (OD) at 205, 235, 270, 280 and 320 nm, respectively. The greatest RNA absorbance should be observed at 260 nm [40]. RNA eluate is considered free from protein and carbolic acid contamination if OD_{260}/OD_{280} (extinction coefficient R_1) is within 1.8–2.2 and is approximately 2.0. Lower readings of R_1 suggest the presence of DNA, protein and residual phenolic compounds in the sample. Higher R_1 readings are indicative of RNA degradation and the presence of free ribonucleotides. The RNA molecules are considered free of polysaccharides if OD_{260}/OD_{235} (extinction coefficient R_2) is close to 2.000 [40, 43, 44]. If 1% of RNA is replaced with polysaccharide components, R_2 decreases by 0.002. R_2 readings greater than 2.000 may indicate degradation of RNA molecules. The absence of suspended particulate matter in the sample is confirmed if the optical density at 320 nm is close to zero.

Table 2
Parameters required for the validation of real-time qPCR-based methods

Application	Analysis characteristics	Major validation parameters
Research	1. Low throughput 2. A great number of different types of samples	1. Analytical sensitivity 2. Analytical specificity
Diagnostics	1. Limited number of targets 2. High throughput protocols targeted at only a few sample types	1. Analytical sensitivity 2. Analytical specificity 3. Accuracy 4. Repeatability 5. Reproducibility

It is important to test for the level of RNA eluate contamination by genomic DNA, as well as to record the cut-off criteria for admissible levels of such contamination in the test protocol. It is required to record information on whether the RNA sample was treated with DNase, as well as to record the results of comparing the C_q obtained with positive control and no reverse transcriptase control for each target nucleic acid [20].

Assessment of RNA integrity in the eluate. To assess the integrity of RNA and the absence of DNA contamination, horizontal gel electrophoresis of denatured RNA is performed, which gives a clearly distinguishable band of nucleic acid molecules without apparent extraneous polynucleotide fragments [45]. Electrophoresis is performed in a thin 0.5% agarose gel prepared using agarose E and 1× RNase-free running buffer, in a voltage gradient of 1–2 V/cm of gel for 45 min. For RNA color-coding after electrophoresis, the gel is dyed with a dye solution with ethidium bromide concentration of 0.4 μg/mL in 25 mM tris-HCl (pH 9.0) for 50–60 minutes. RNA bands are observed using an ultraviolet transilluminator [45, 46].

DNA sample assessment. It is important to assess the extent of DNA degradation for forensic applications, i.e., in cases in which harsh environmental conditions at scenes of crimes may have degraded the chemical structure of DNA. It should be noted that DNA molecules are more stable and less susceptible to degradation than RNA. For this reason, the DNA purity and integrity is assessed much less often. At the same time, there is a general rule for DNA samples, to routinely use dilutions of nucleic acids to demonstrate that observed decreases in qualification cycles or copy numbers are consistent with the anticipated result [47].

Requirements for the reverse transcription reaction. When handling RNA samples, a reverse transcription step

introduces substantial variation into a qPCR assay [48, 49]. Hence the following information should be given in detail in the test protocol:

– reagent composition;
– temperature-time parameters of the reverse transcription step;
– RNA amount in the collected sample;
– priming strategy;
– enzyme type;
– reaction volume.

It is recommended that the reverse transcription step be carried out in duplicate or triplicate and that the total RNA concentration be the same in every sample [49].

Requirements for qPCR. To develop a method based on quantitative PCR, the author must know the following information:

– database accession numbers of each target and reference genes (for example, GenBank, WIPO Sequence and others);
– the exon locations of each primer and any probe;
– the sequences and concentrations of each oligonucleotide, positions of dyes and/or modified bases used in the probe;
– published sequences of primers and probes (since the amplification efficiency largely depends on the oligonucleotides used);
– the concentration and identity of the polymerase;
– the amount of template (DNA or cDNA) in each reaction;
– the Mg^{2+} concentration;
– the exact chemical composition of the buffer (salts, additives, hydrogen ion concentration);
– total volume of the components for one reaction;
– PCR machine calibration certificate;
– data on thermal cycling time and temperature conditions;
– information about the degree of transparency of the plasticware used and the material from which they are made (because different plastics exhibit substantial differences in fluorescence reflection and sensitivity) [50, 51].

Requirements for the calculation of oligonucleotide primers and probes. The structure of the target nucleic acid (for example, stem and loop secondary RNA) has a significant impact on the efficiency of reverse transcription and the PCR [52]. Therefore, the positions of primers, probes and PCR amplicons must take the folding of RNA templates into consideration.

To develop qPCR-based methods it is useful to use such tools for oligonucleotide specificity assessment *in silico*, as BLAST[2], etc. Any appreciable homology to pseudogenes or other unexpected targets should be documented and provided as aligned sequences for review.

Another important requirement is that specificity must be validated empirically with direct experimental evidence (electrophoresis gel, melting profile, DNA sequencing, amplicon size, and/or restriction enzyme digestion) [53, 54].

An important primer characteristic is their size, responsible for the reaction specificity. As a rule, the length of primers is from 17 to 35 b.p., but there may be exceptions (a larger length is acceptable). The length of the nucleotide chain affects the melting temperature. Melting temperature (hybridization, dissociation, T_m) is the temperature at which half of the oligonucleotides in solution are in the double-stranded state and half single-stranded. T_m depends on the hydrogen bonding between the nucleotides of the primer and the DNA molecule it is hybridized with. There are different approaches to this indicator assessment. Traditionally the melting temperature is defined as the inflection point on the hybridization graph, which corresponds to the maximum modulus of the first derivative. Less often, the temperature at which 50% of the fluorescent signal is reached, is considered to be primer dissociation temperature [17, 54–56]. It should be noted that there are calculated and true values of the melting temperature. The calculated value is obtained using a number of formulas, and this value is theoretical, the true value is obtained by molecular biological experiment. In practice, various formulas or bioinformatic tools are used to solve this problem [57, 58].

Thermodynamic calculations in the context of the base energy are performed as described by K. J. Breslauer et al. [59], but using the values published by N. Sugimoto et al. [60]. RNA thermodynamic properties are taken from the publication of T. Xia et al. [61]. Melting temperature calculations are based on the thermodynamic relationship between entropy, enthalpy, free energy and temperature: $\Delta H = \Delta G + T\Delta S$, where ΔH is enthalpy; ΔG is Gibbs energy; T is absolute temperature (K); ΔS is entropy.

The change in entropy (order or a measure of the randomness of the oligonucleotide) and enthalpy (heat released or absorbed by the oligonucleotide) are directly calculated by summing the values for nucleotide pairs obtained by N. Sugimoto et al. [60]. The relationship between the free energy and the concentration of reactants and products at equilibrium is given by the following formula:

$$\Delta G = RT\ln\left(\frac{[DNA \times primer]}{[DNA]\,[primer]}\right),$$

where R is the gas constant (8.31 [J/mol × K]); T is the absolute temperature (K); ln is the natural logarithm; [DNA × primer] is the concentration of the bound DNA × primer complex; [DNA] is the concentration of unbound DNA target sequence; [primer] is the concentration of unbound primer.

Substituting for ΔG gives:

$$\Delta H = T\Delta S + RT\ln\left(\frac{[DNA \times primer]}{[DNA]\,[primer]}\right).$$

The absolute temperature is expressed using the following equation:

$$T = \frac{\Delta H}{\Delta S + R\ln\left(\frac{[DNA \times primer]}{[DNA]\,[primer]}\right)}.$$

We can assume that the concentration of DNA and the concentration of the DNA × primer complex are equal (that is, the concentration of primer is in excess of the target DNA and the melting temperature is where the concentration of bound and unbound DNA are at equilibrium), so this simplifies the equation considerably. It has been determined empirically that there is a 5 kcal free energy change (according to N. Sugimoto et al. [60]) during the transition from single stranded to B-form DNA. This

[2] Basic Local Alignment Search Tool (BLAST). Available at: https://blast.ncbi.nlm.nih.gov/Blast.cgi .

represents the helix initiation energy. Finally, adding an adjustment for salt gives the equation:

$$T = \frac{\Delta H - 3{,}4\ \frac{\text{kcal}}{^{\circ}\text{K mole}}}{\Delta S + R \ln\left\{\frac{1}{[\text{primer}]}\right\}} + 16{,}6 \log_{10}([Na^+]),$$

where T – absolute temperature (K); ΔH – enthalpy; kcal – energy; mole – amount of substance; ΔS – entropy; R – gas constant (8.31 [J/mol × K]); ln – natural logarithm; [primer] – concentration of unbound primer; $\log_{10}$ – decimal logarithm (lg); $[Na^+]$ is the concentration of sodium cations.

An adjustment constant for salt concentration is not needed, since the various parameters were determined at 1 M NaCl, and the log of 1 is zero. The thermodynamic calculations assume that the annealing occurs at pH 7.0. The T_m calculations assume the sequences are not symmetric. The oligonucleotide sequence should be at least 8 bases long to give reasonable T_m. The accuracy of the calculation decreases after 20–25 nucleotides since the equations and parameters were defined with oligonucleotides in the size range of 14–20 nucleotides. Monovalent cation concentrations (either Na^+ or K^+) should be between 0.01 and 1.00 M. The melting temperature of oligonucleotides can also be calculated using bioinformatics software, for example Primer3plus[3] [50].

If a theoretically calculated melting temperature differs significantly from the true one, this is not critical, since the annealing temperature (T_a) and the ratio of oligonucleotide T_m to each other and their correspondence to this temperature in the thermocycler program are more important for the test system. In this context, the investigators test several conditions for the amplification reaction, namely the annealing temperature of primers and select the optimal one [15, 48, 50].

Many different algorithms are used to theoretically determine the hybridization temperature, but none of them gives 100% confidence in obtaining the true value. The recommended melting temperature for oligonucleotides is from 55 to 75 °C. The hybridization temperatures of the forward and reverse primers should differ by no more than 5 °C. It should be noted that the greater the G+C content and the length of the oligonucleotide, the higher the T_m [22].

The transcripts of most genes in multicellular organisms are alternatively spliced, and these splicing variants specify alternative protein isoforms. It is known that there are differences in splicing patterns in different tissues or at different developmental stages. Consequently, single exon-based RT-qPCR assays may detect a number of splice variants, whereas intron-spanning primers may be more selective but may miss some splice variants altogether [45].

In this context the development of qPCR-based methods requires adherence to the following rules (and exceptions) when determining target genes for mRNA:

1) the use of an qPCR assay that simply targets one or at most two exons of an mRNA is no longer sufficient to describe the expression level of a particular gene;

2) sequence information for primers must be provided together with an assessment of their specificity with respect to known splice variants and single-nucleotide polymorphism positions [54]. For primer sets selected from the RTprimerDB database [56], this is easily done by consulting the web-site[4], that contains all the relevant information. It is not recommended to provide results that have been confirmed only *in silico*;

3) it must be remembered that detection of the presence of an mRNA provides no information on whether that mRNA will be translated into a protein or, indeed, whether a functional protein is translated at all [36].

Requirements for qPCR controls and calibrators. In addition to controls performed at the stages of nucleic acid extraction and reverse transcription reaction, additional controls and/or quantitative calibrators are also required for the development of quantification techniques [27, 36].

No template control. It is recommended to use a no template control (NTC), which allows screening for contamination and can also distinguish unintended amplification products (e.g., primer dimers) from the intended PCR products [48].

The NTC use is based on the possibility of performing many separate PCR reactions all together in one reaction for different DNA fragments (multiplex PCR) [62, 63]. For example, to control the amplification performance, two PCRs can be carried out simultaneously in one test tube. In one of these reactions, the target DNA (or cDNA) fragment is accumulated, and in the other, specially included DNA is amplified (usually a plasmid DNA fragment). Inclusion of NTC into the sample before the nucleic acid extraction, makes it possible to monitor the efficiency of all analytical stages.

For reverse transcription PCR, it is recommended to use NTC. This is a specially designed RNA product added to each tested sample at the stage of sample preparation (exogenous internal control), which goes through all stages of polymerase chain reaction. At the PCR detection stage, NTC allows us to judge the quality of the amplification result in general. It is added immediately before the nucleic acid extraction. If during PCR analysis a signal is detected in the NTC, this means the result of the PCR is reliable, otherwise the PCR result will be invalid [2, 52].

NTCs should be included on each plate or batch of samples, and conditions for data rejection be established. For example, NTCs with quantification cycle values 40 could be ignored if the C_q for the lowest concentration is 35 [9].

For optimal PCR results, physically separated working places for template preparation before PCR and setting up PCR reactions are recommended [17].

Positive controls in the form of nucleic acids extracted from experimental samples are useful for monitoring assay variation over time and are essential when calibration curves are not performed in each run.

Quantification calibrators may be the following:

– purified target molecules, such as synthetic RNA or DNA oligonucleotides spanning the complete PCR amplicon;

– plasmid DNA constructs;

– cDNA cloned into plasmids;

– RNA transcribed *in vitro*;

– reference RNA pools;

– RNA or DNA from specific biological samples or internationally recognized biological standards.

[3] Primer3Plus. Available at: https://www.primer3plus.com.

[4] RTprimerDB. Available at: http://www.rtprimerdb.org.

Suspension dilutions should be carried out into defined concentrations. Serial dilutions of a particular template can be prepared as stock solutions that resist several freeze-thaw cycles. A fresh batch should be prepared when a C_q shift of 0.5–1.0 is detected. Alternatively, solutions for calibration curves can be stored for not longer than a week at (2 ± 1) °C [12].

For diagnostic assays, the qPCR should include an independently verified calibrator, if available, that lies within the linear interval of the assay.

PCR negative control. In addition to positive control at the stage of the amplification, it is absolutely necessary to use negative control, which most often is deionized water, not contaminated by extraneous nucleic acids, enzymes, microorganisms [35].

Nucleic acid extraction controls. Positive and negative controls to be used during RNA/DNA extraction are also recommended [55].

Assay performance. The following assay performance characteristics must be determined when qPCR method is developed: PCR efficiency, linear dynamic range, limit of detection, and precision.

PCR efficiency. Robust and precise qPCR assays are usually correlated with high qPCR efficiency. qPCR efficiency is particularly important when reporting mRNA concentrations for target genes relative to those of reference genes.

The C_q ($\Delta\Delta C_q$) method is one of the most popular means of determining differences in concentrations between samples and is based on normalization with a single reference gene. The difference in C_q values (ΔC_q) between the target gene and the reference gene is calculated, and the C_qs of the different samples are compared directly. The 2 genes must be amplified with comparable efficiencies for this comparison to be accurate [21, 43]. As an example, the figure shows the fluorescence accumulation curves for FMDV cDNA suspensions with different template concentrations (from 10 fg to 300 ng/mL).

PCR amplification efficiency must be established by means of calibration curves, because such calibration provides a simple, rapid, and reproducible indication of the mean PCR efficiency, the analytical sensitivity, and the robustness of the assay. Amplification efficiency should be determined from the slope of the log-linear portion of the calibration curve using the following formula: $E = 10^{-1/k} - 1$, where k is the slope of the dependency graph between the logarithm of the initial template concentration (the independent variable), plotted on the x-axis and C_q (the dependent variable), plotted on the y-axis.

An E value of 1.00 (or 100%) indicates that the amount of product doubles with each cycle (theoretically).

The means of estimated PCR efficiencies and slope values should be recorded in the qPCR protocols. Differences in PCR efficiency will produce calibration curves with different slopes. As a consequence, differences between the C_q values of the targets and the references will not remain constant as template amounts are varied, and calculations of relative concentrations will be inaccurate, yielding misleading results [12, 35, 54].

Attention should be paid to the fact that if C_q values are close to 40, low efficiency is highly probable or template content is too low for analytical sensitivity [36].

qPCR linear dynamic range. The dynamic range of the quantitative analysis (the range of the template contents in the tested samples) over which a reaction is linear must be described [39]. The dynamic range should cover at least 3 orders of magnitude. The calibration curve's linear interval must include the interval for the target nucleic acids being quantified [14].

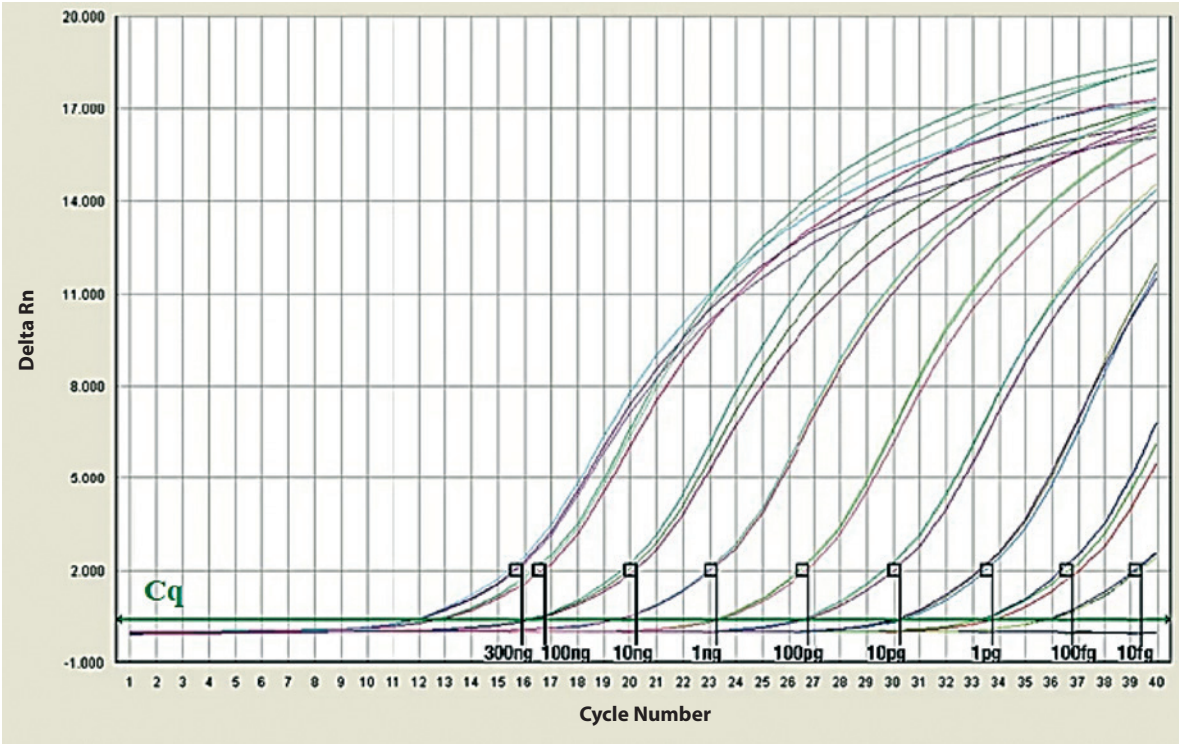

Fig. Fluorescence accumulation curves obtained by testing of FMDV cDNA suspensions at analyte concentrations within 10 fg to 300 ng/mL

Because lower limits of quantification are usually poorly defined, the variation at the lowest concentration claimed to be within the linear interval should be determined. Correlation coefficients (r^2 values) must be reported, and confidence intervals (CIs) should be provided through the entire linear dynamic range [64].

The limit of detection (LOD) is the minimum target concentration in a sample that can be detected with an acceptable level of confidence. Thus LOD is defined as the lowest concentration at which 95% of the positive samples are detected. In other words, within a group of replicates containing the target at concentrations at the LOD, no more than 5% failed reactions should occur. Low-copy qPCRs are stochastically limited, and LODs of 3 copies per PCR are not possible. If multiple reactions are performed, however, accurate quantification of lower concentrations can be obtained via digital PCR [18, 22].

The smallest target concentration is calculated by the formula: $LOD = 3.3 \times S_b/k$, where S_b is the standard deviation of the detected signal, which corresponds to the intercept standard deviation (b); k is the tangent of the slope [65, 66]. The intercept is calculated by analyzing a certain number of model samples with known target concentrations.

The limit of quantification (LOQ). The lowest analyte concentration that can be quantitatively detected with a stated accuracy and precision of the validated technique which is calculated using the formula: $LOQ = 10 \times S_b/k$ [66]. The obtained LOQ value should be subsequently validated by direct experimental evidence using a suitable number of samples known to be near to the LOQ. The analysis should be performed in at least five replicates. The results of the test are considered reliable at $p < 0.05$ [66–69].

Linearity. The linear relationship between analyte concentration and response should be evaluated across the working range of the analytical procedure for ≥ 30 samples, using different analyte concentrations in at least three replicates. The obtained data are processed by calculation of a regression line by the method of least squares: $y = k \times x + b$, where k is the angular coefficient; b is the intercept. The reliability of the analysis results is confirmed by calculating the correlation coefficient (r^2), which should be ≥ 0.99 [69].

Accuracy. To determine the accuracy, samples with known analyte amounts are analyzed by quantitative PCR. The data are given as a linear equation for the experimental values (y) and the reference (x) values of the analyte: $y = k \times x + b$. For the resulting function, the hypotheses about the equality of the slope (k) to one and the equality of the intercept (b) to zero are verified. If these hypotheses are true with 0.05 reliability, the validated technique gives error-free results [64].

Precision. To assess the precision of the qPCR test kits being developed against repeatability and reproducibility, it is required to calculate absolute and relative measures of variation.

Absolute quantification. The range of variation (R) is defined as the difference between the maximum and minimum values of the quantification cycle: $R = Cq_{max} - Cq_{min}$. The individual linear deviation (d_i) is calculated using the following formula $d_i = |Cq_i - Cq_m|$. The mean linear deviation (d_m) is calculated as the arithmetic mean of individual linear deviations: $d_m = \Sigma|d_i| / N$, where d_i is the individual linear deviations of the quantification cycles; N is the population size. The dispersion (δ^2) of the values is estimated using the following formula $\delta^2 = (\Sigma d_i^2) / N$. To characterize C_q variations, the root-mean-square deviation (δ) is calculated using the following mathematical model $\delta = \sqrt{(\delta^2)}$ [64–66, 70].

Relative quantification. Relative variation coefficient (V_R) is calculated by the formula $V_R = R / C_{tm} \times 100$. The linear coefficient of variation (C_d) is calculated using a mathematical model $C_d = d_m / C_{tm} \times 100$. To assess the dispersion of individual C_p values, the coefficient of variation (C_δ) is determined by the formula: $C_\delta = \delta / C_{tm} \times 100$ [17]. The method is considered reliable at $C_\delta < 2\%$ for repeatability and at $C_\delta < 3\%$ for reproducibility [64–67].

The statistics of the diagnostic tests. The main statistical indices of the diagnostic tests are: diagnostic sensitivity (DSe), diagnostic specificity (DSp), k (Cohen's kappa coefficient), positive predictive value (PPV), negative predictive value (NPV). To measure them, the following test results are needed: a – true positive samples; b – false negative samples; c – false positive samples; d – true negative samples.

Diagnostic sensitivity and specificity are calculated by the formulas: Dse = a / (a + b) and DSp = d / (c + d), and expressed as a percentage.

The Cohen's kappa index value (k) is used to measure the of inter-rater agreement on any two tests. Cohen's kappa index value is calculated using the following formula: k = (Pr (a) – Pr (e)) / (1 – Pr (e)), where Pr (a) – relative observed agreement; Pr (e) – hypothetical probability of chance agreement.

The probability of the positive result in the test is calculated by the formula: PPV = (Dse × prevalence) / ((Dse × prevalence) + (1 – DSp) × (1 – prevalence)), where prevalence is the number of events, in this variant, positive samples being detected from truly positive ones at a certain moment. This value should aim at 100%.

The probability of a negative test result when testing true negative samples is calculated by the formula: NPV = DSp × (1 – prevalence) / ((1 – DSe) × prevalence + DSp × (1 – prevalence)). This value should aim at 100% [64–68, 70].

REQUIREMENTS FOR qPCR DATA ANALYSIS

General requirements. Data analysis includes an examination of the raw data, an evaluation of their quality and reliability, and the generation of reportable results [2, 71]. When developing qPCR, it is necessary to specify up-to-date information in the protocol:

– methods of data analysis and confidence estimation;
– specification of the software used;
– methods of identifying outliers;
– statistical and validation methods used to evaluate variances (e.g., 95% CIs) and presentation of the corresponding concentrations or C_q values for precision analysis for repeatability and reproducibility [67, 70].

Normalization of qPCR data. Normalization is an essential component of a reliable qPCR assay because this process controls for variations in extraction yield, reverse-transcription yield, and efficiency of amplification, thus enabling comparisons of mRNA concentrations across different samples.

The use of reference genes as internal controls is the most common method for normalizing qPCR data. Normalization involves reporting the ratios of the mRNA concentrations of the genes of interest to those of the reference genes. Reference gene mRNAs should be stably expressed, and their abundances should show strong correlation with the total amounts of mRNA present in the samples.

It should be noted, that normalization against a single reference gene is not acceptable unless the investigators present clear evidence for the reviewers that confirms its invariant expression under the experimental conditions described [25]. The optimal number and choice of reference genes must be experimentally determined. This process is described in detail in the publications of J. Vandesompele et al. [72], C. L. Andersen et al. [73].

CONCLUSION

The analysis of publications and international protocols presents general requirements and recommended rules for the development of qPCR-based methods, compliance with which will allow reviewers to evaluate the work and other investigators to reproduce it.

In accordance with current approaches (in particular, with the MIQE guidelines – a database of checklists for developed qPCR techniques[5]), for the presentation of materials, a qPCR data markup language (RDML) is used, which is a structured and universal standard of results for the exchange of PCR quantitative data. According to these principles, the method should contain sufficient data to ensure correct interpretation and repeatability. The data standard is a flat text file in Extensible Markup Language (XML) and enables transparent exchange of annotated qPCR data between instrument software and third-party data analysis packages, between colleagues and collaborators, and between authors, peer reviewers, journals and readers.

In summary, the purpose of these guidelines is 3-fold:

1. To enable authors to design and report qPCR experiments that have greater inherent value.

2. To allow reviewers and editors to measure the technical quality of submitted manuscripts against an established yardstick.

3. To facilitate easier replication of experiments described in published studies that follow these guidelines. As a consequence, investigations that use this widely applied technology will produce data that are more uniform, more comparable, and, ultimately, more reliable.

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Method of obtaining and storing hyperimmune anthrax serum

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SUMMARY
Anthrax is a highly dangerous disease of animals and humans caused by the spore-forming bacterium *Bacillus anthracis*. Currently, the disease is widespread in many countries of the world. Many regions of the Russian Federation are anthrax-endemic. A large number of anthrax treatment, diagnosis and prevention tools are developed using hyperimmune serum. Currently known commercial hyperimmune sera are produced by 2-month long immunization of horses, which is a long and expensive process. This suggests the need to develop faster and cheaper ways to produce anti-anthrax hyperimmune sera; such possible ways became the objective of this study. A live culture of *Bacillus anthracis* 55-VNIIVViM vaccine strain, used to produce live vaccines against animal anthrax, was used in the experiments. Rabbits were used as animal models. Based on the findings the method of rabbit immunization was selected. The optimal method included intravenous injection of the antigen in increasing amounts according to the following scheme: injection I – 0.5 cm^3; injection II – 1 cm^3; injection III – 2 cm^3 at a dose of 100 million mc/animal in 1 cm^3, with 4-day interval between injections. This scheme made it possible to produce the serum with a high antibody titer equal to 14 log_2. For long-term storage of the serum produced, the freeze-drying modes were optimized, giving 2% residual moisture content of the finished product. The analysis of the freeze-dried serum storage terms showed that the initial activity and physico-chemical properties of the product are maintained for 30 months.

Keywords: *Bacillus anthracis*, immunization, serum, antigen, anthrax, antibodies

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Способ получения и хранения гипериммунной сибиреязвенной сыворотки

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РЕЗЮМЕ
Сибирская язва – особо опасная инфекционная болезнь животных и человека, вызываемая спорообразующей бактерией *Bacillus anthracis*. В настоящее время данное заболевание широко распространено во многих странах мира. Некоторые регионы Российской Федерации являются эндемичными по сибирской язве. Большое число средств терапии, диагностики и профилактики сибиреязвенной инфекции разрабатываются на основе гипериммунных сывороток крови. Известные в настоящее время коммерческие сыворотки крови получают путем гипериммунизации лошадей, длящейся в течение 2 мес. и представляющей длительный и дорогостоящий процесс. Данный факт свидетельствует о необходимости разработки более быстрых и дешевых способов получения гипериммунных противосибиреязвенных сывороток крови, что и явилось целью работы. В опыте использовали живую культуру вакцинного штамма 55-ВНИИВВиМ *Bacillus anthracis*, который применяется в России для создания живых лекарственных препаратов против сибирской язвы животных. В качестве модели для получения сывороток крови были выбраны кролики. В результате проведенной работы подобран способ гипериммунизации кроликов, включающий внутривенное введение антигена в нарастающем объеме по схеме: I инъекция – 0,5 $см^3$; II инъекция – 1 $см^3$; III инъекция – 2 $см^3$ в дозе 100 млн м. к./гол. в 1 $см^3$ с интервалом между введениями 4 сут. Указанная схема дала возможность получить сыворотку крови с высоким титром антител, равным 14 log_2. Для долгосрочного хранения полученной сыворотки отработан режим ее лиофилизации, позволивший достичь остаточной влажности готового препарата в 2%. При изучении длительности хранения лиофилизированной сыворотки было установлено, что исходная активность и физико-химические свойства препарата сохраняются в течение 30 мес.

Ключевые слова: *Bacillus anthracis*, гипериммунизация, сыворотка, антиген, сибирская язва, антитела

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INTRODUCTION

Anthrax is a deadly infectious disease caused by the spore-forming bacterium *Bacillus anthracis* [1, 2]. To date, outbreaks of this infection continue to be registered both among animals and among people in many countries of the world [3–5]. In addition, there is a constant risk of the disease introduction from other countries [6]. According to the World Organization for Animal Health, in 2019, anthrax was registered in 46 countries of the world, in 2020 – in 23, in 2021 – in 22, in 2022 in 16 countries [7]. Many regions of the Russian Federation are endemic for anthrax [8, 9]. In most cases, the disease is sporadic, involving a small number of animals. However, the anthrax outbreak that occurred in Yamal demonstrates the risk of complicated situation [10, 11]. This fact makes the development of diagnostic, prevention and therapeutic tools to control this highly dangerous disease an urgent matter.

The test kit for determination of antibody titers in sera of anthrax-vaccinated animals by indirect immunohemagglutination test (IHA) [12] shall be standardized in the process of its development, including determination of the antigen activity. Moreover, in addition to determination of antibody levels in vaccinated animals, a confirmation test using known positive control serum is required. Subsequently, this serum will be included as the reagent for the developed test kit.

Currently known commercial hyperimmune sera are obtained by long-term (for 2 months) immunization of horses [13]. This suggests the need to search for faster and cheaper ways of hyperimmune anthrax serum production. The novelty of this study consists in the proposal of a rabbit immunization scheme, which allows obtaining of highly active anthrax hyperimmune serum in a short time.

In this regard, the goal was to develop a method for hyperimmune anthrax serum production, which will serve as a control for serological testing to determine the antibody level in animals vaccinated against anthrax.

MATERIALS AND METHODS

Strain. *B. anthracis* 55-VNIIVViM (pX01+/pX02–) vaccine strain was used as an immunizing antigen.

Nutrient media. For the cultivation of *B. anthracis*, meat-peptone agar (MPA) and meat-peptone broth (MPB), 5% blood agar, 12% gelatin, skimmed milk and Hottinger broth produced by the FSBSI "FCTRBS-ARRVI" (Russia) were used.

Laboratory animals. In order to obtain hyperimmune anthrax serum, 2.5–3.0 kg chinchilla rabbits after a 30-day quarantine, were used. Fifteen animals in total were divided into three groups (5 animals per each).

Experiments on animals were conducted in compliance with the requirements of Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes.

Reagents and test kits. 0.9% sodium chloride solution (Grotex LLC, Russia) was used to harvest the culture from the agar surface, as well as a diluent for IHA test.

For IHA test, the anthrax antigenic erythrocyte diagnosticum was used (FSBSI "FCTRBS-ARRVI", Russia).

Methods. The strain biological properties were tested according to MG 4.2.2413-08 "Laboratory diagnostics and detection of the anthrax causative agent"[1].

Suspension for immunization of laboratory animals with concentrations of 100 and 500 million mc per 1 cm^3, depending on the scheme, was prepared from a strain grown on MPA at 37 °C for 2 days.

The antibody titer increase was monitored by IHA test every 3 days in U-bottom plates (OOO "MiniMed", Russia) before the next administration of the antigen, according to the manufacturer instructions for the diagnosticum used. 0.2 cm^3 of 0.9% sodium chloride solution was added to all the wells of the plate using a multichannel pipette. 0.2 cm^3 of the obtained and control (negative) serum were added to the first wells of the rows, and then two-fold serial dilutions were performed. After preparation of the corresponding serum dilutions, 0.05 cm^3 (50 μL) suspensions of anthrax antigenic erythrocyte diagnosticum were added into all wells. The plates were carefully shaken to mix the reaction components and left at 10–20 °C for 1.5–2.0 hours. After exposure, the results were read.

Blood was collected from producing animals totally from the heart. Blood was collected into sterile glass

[1] MG 4.2.2413-08 Laboratory diagnostics and detection of the anthrax causative agent: methodical guidelines. Moscow: Rospotrebnadzor Federal Center of Hygiene and Epidemiology; 2009. 69 p. Available at: https://files.stroyinf.ru/Data2/1/4293752/4293752010.pdf.

cylinders with 0.9% sodium chloride solution pre-moistened walls, placed in a thermostat for coagulation for 45–60 minutes, then the clot was separated from the cylinder walls by circular movements using a glass sterile stirring rods and put in the refrigerator at 4 °C for 24 hours. The separated serum was decanted from the clot using a sterile pipette after activity testing.

The obtained sera were freeze-dried using LZ-9.2 freeze-dryer (Frigera, Czech Republic).

The obtained serum was evaluated for the following parameters: appearance, colour, impurities, solubility, activity after during long-term storage, moisture content of the freeze-dried product.

The appearance, colour, impurities were checked visually.

To determine the solubility, 1 cm^3 of 0.9% sodium chloride solution was added to the bottles with serum. After that, the bottles were shaken and dry mass dissolution process was monitored.

The activity of the obtained freeze-dried serum during long-term storage at 4 °C was determined after 3, 6, 9, 12, 15, 18, 24, 30, 36 and 42 months by IHA test using erythrocyte antigenic anthrax diagnosticum. Serum stability values were calculated in $\log_2$.

Determination of moisture content of lyophilized serum was performed according to GOST 24061-2012[2].

The obtained data were statistically processed using Mann – Whitney U-test. The test results are presented as $M \pm S_D$, where M is the average value, S_D is the standard deviation. Differences were considered statistically significant at $p < 0.01$ (after adjustment for the number of comparisons) [14].

TEST RESULTS

The aim of first working stage was to study the basic biological properties of *B. anthracis* 55-VNIIVViM strain.

After inoculation of the strain on MPA and MPB and cultivation for 24 hours, cultural, morphological, tinctorial properties were studied, motility was recorded.

Flat, dull greyish, rough (R-form) colonies were observed on MPA (Fig. 1A) with darkened centre and fringed edge with put out curled protrusions (Fig. 1B).

24 hours after inoculation in MPB, the medium remained transparent, a loose cotton wool-like sediment formed at the bottom (Fig. 1C). When shaking the test tube, the broth did not become cloudy, the sediment was hardly broken into small flakes.

The broth culture was smeared, Gram-stained and microscoped. Typical chains consisting of anthrax gram-positive rods were observed in the smears (Fig. 1D).

The analysis of the basic biological properties of *B. anthracis* 55-VNIIVViM strain showed that its properties are typical for its species (Table 1).

The aim of the next working stage was the search for optimal immunization scheme for laboratory animals to obtain active hyperimmune anthrax serum. The prepared antigen was administered using three schemes: 1) in increasing volumes intravenously: I injection – 0.5 cm^3; II injection – 1.0 cm^3; III injection – 2.0 cm^3 at a dose of 100 million mc/animal per 1 cm^3 with 4 day-interval between injections; 2) once at a dose of 500 million mc/animal per 1 cm^3 intravenously; 3) twice at a dose of 100 million mc/animal per 1 cm^3 intradermally along the spinal column into five points on each side with a 5 day-interval between injections.

In the process of rabbit immunization, the dynamics of the antibody titer growth was monitored by blood collection and IHA test every 3 days. The use of 55-VNIIVViM vaccine strain as an antigen made it possible to obtain an active immune anthrax serum. The results of rabbit hyperimmune serum activity testing after using three schemes of hyperimmunization are shown in Figure 2.

During the entire period of laboratory animal hyperimmunization, an increase in the antibody titer was observed.

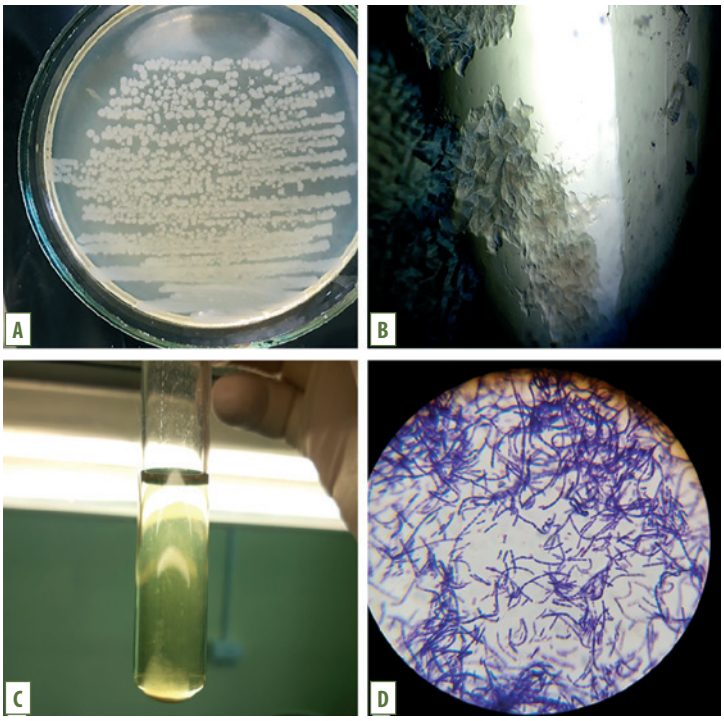

Fig. 1. B. anthracis 55-VNIIViM morphology:
A – R-shaped colonies on MPA;
B – B. anthracis 'curly hair' colonies (8 × 40);
C – B. anthracis typical 'cotton wool'-like growth in MPB;
D – morphology of Gram-stained B. anthracis cells

Table 1
Major biological properties of *B. anthracis* 55-VNIIVViM strain

No.	Indicator (property)	*B. anthracis* 55-VNIIVViM properties	*B. anthracis* 55-VNIIVViM according to Bergey's manual
1	Motility	–	–
2	Hemolytic properties	–	–
3	Proteolytic properties: 12% gelatin skimmed milk	 + +	 + +
4	Capsule formation	–	–
5	Susceptibility to penicillin	+	+
6	Spore formation	+	+

"+" – yes; "–" – no.

[2] GOST 24061-2012 Medicine remedies biological lyophilized for veterinary use. Method for determination mass moisture. Available at: https://docs.cntd.ru/document/1200103299.

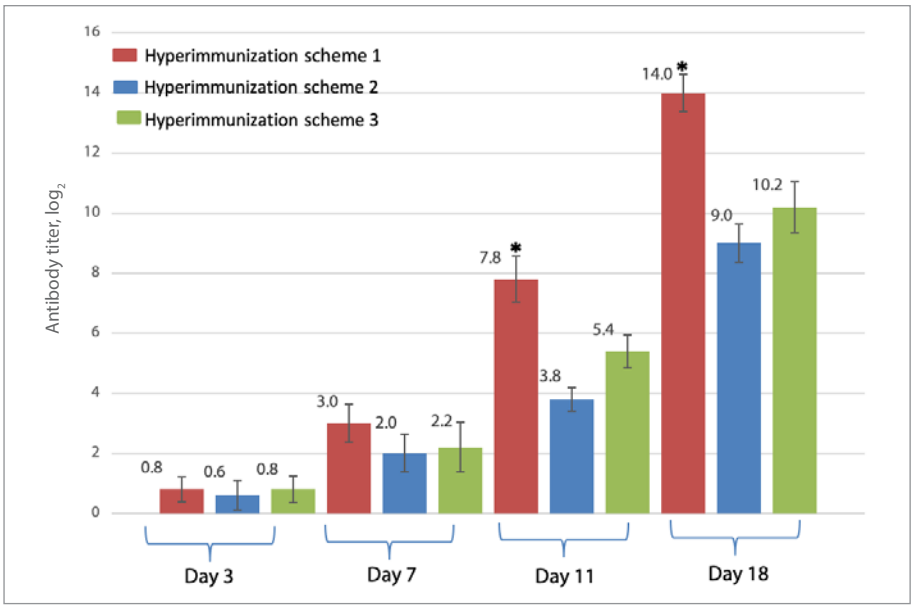

Fig. 2. Specific antibody titer dynamics in sera of hyperimmunized rabbits (statistically significant difference, $p < 0.01$)*

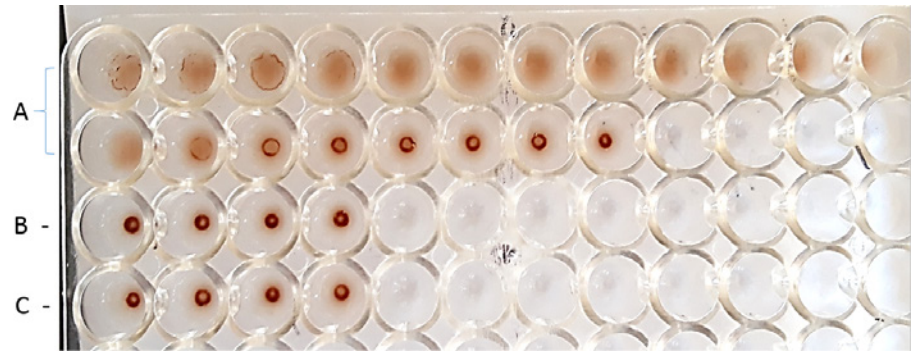

Fig. 3. Activity of produced hyperimmune rabbit sera tested by IHA test using RBC anthrax antigen: A – reaction to produced hyperimmune serum (antibody titer 14 $\log_2$); B – reaction to normal serum (serum of non-immunized animal); C – reaction to saline solution

The highest level of specific antibodies in rabbit sera was observed after the first scheme immunization: after antigen injection by day 18, the titer reached the value of 14 $\log_2$, which is equivalent to 1:16384 serum dilution (Fig. 3). Therefore, this scheme was used for the development of diagnostic products.

Sera used as components of diagnostic test kits remain active better and are more easily transported when freeze-dried ones [15]. Therefore, tests were conducted to choose the optimal lyophilization conditions. For this purpose, sera was freeze-dried using two different modes (Table 2).

When two freeze-drying modes were compared, it was found that mode 1 makes the serum homogeneous of creamy white colour (Fig. 4A). Mode 2, in which freeze-drying process took a longer time, made the product excessively dry (Fig. 4B).

Table 2
Different modes of serum freeze-drying

Days	Mode 1		Mode 2	
	time	stage	time	stage
1	11:30	1. Turning on the freeze-drier	8:00	1. Turning on the freeze-drier
	12:00	2. Loading of sera into the freeze-drier at –35 °C	8:30	2. Loading of sera into the freeze-drier at –35 °C
	16:00	3. Turning off the freezing at –37 °C	16:00	3. Turning off the freezing at –36 °C
2	8:00	1. Turning on the heating (t in the chamber +10 °C)	8:00	1. Turning on the heating (t in the chamber +10 °C)
	11:00	2. Turning off the freeze-drier (t in the chamber +35 °C)	11:00	2. Turning off the freeze-drier (t in the chamber +35 °C)

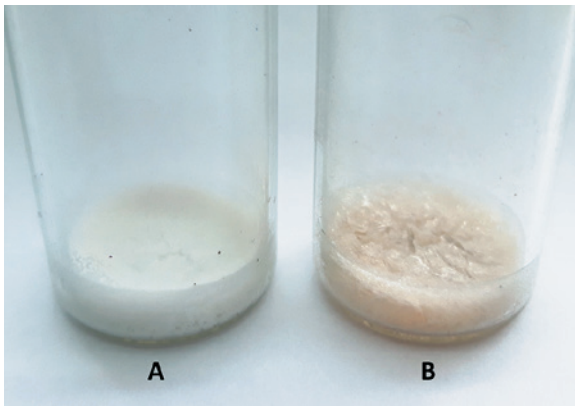

Fig. 4. Visual evaluation of freeze-dried hyperimmune serum: A – freeze-drying mode 1; B – freeze-drying mode 2

Table 3
Physico-chemical and biological parameters of freeze-dried serum

Parameter	Serum characteristics
Appearance	Dry solid matter in the form of a tablet
Colour	Creamy white colour
Solubility	When 1 cm^3 of 0.9% NaCl solution was added, it dissolved within 1–3 minutes
Activity	Agglutinates antigenic erythrocyte anthrax diagnosticum in 1:16384 dilution
Moisture content, %	2.0

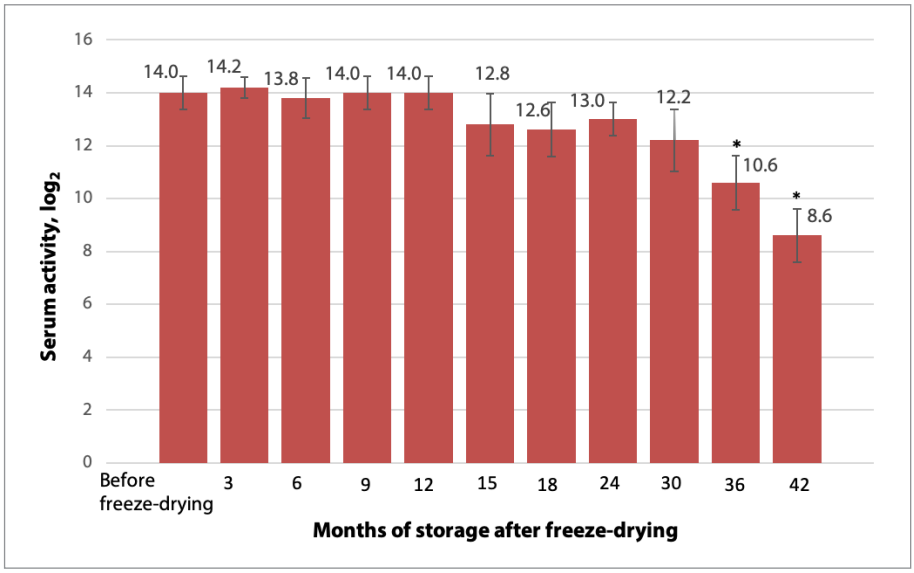

Fig. 5. Activity of freeze-dried hyperimmune rabbit serum during long-term storage at 4 °C (statistically significant difference, $p < 0.01$)*

After lyophilization, the quality control of the obtained serum was performed. The physico-chemical and biological parameters of the serum are given in Table 3.

The stability testing of the obtained freeze-dried serum during long-term storage at 4 °C revealed that the initial titer persists for at least 30 months, followed by a decrease in activity to 10 $\log_2$ 36 months after lyophilization (Fig. 5). Consequently, freeze-drying of rabbit hyperimmune serum makes it possible to maintain high activity of the product for a long time.

DISCUSSION

During the study, rabbit anti-anthrax serum with a high antibody titer was obtained. The search for manufacturing methods of such freeze-dried products is associated with the need to use it as a control of the developed anthrax diagnosticum. Many researchers have demonstrated the effectiveness of using mice, guinea pigs, goats and horses to produce anti-anthrax sera [16–21]. In our experiment, rabbits served as models. This made it possible to obtain a larger volume of serum compared to using of mice or guinea pigs (the amount of serum from one mouse varies within 0.5 mL). Whereas larger animals, such as goats and horses, require higher costs for the keeping infrastructure.

The preparation of mono- and polyclonal globulins and sera against *B. anthracis* antigens is widely described in the scientific literature [22–26]. However, the production of polyclonal sera to the antigens of *B. anthracis* live cells is not discussed in detail. M. Caldwell et al. described the production of equine serum after hyperimmunization with *B. anthracis* Sterne (pX01+/pX02–) strain [21]. In the course of our experiment, we also produced serum against live bacterial cells of *B. anthracis* 55-VNIIVViM (pX01+/pX02–) strain, since it is to be used primarily as a positive diagnostic control and reproduce the properties of sera of immunized animals. The use of live bacterial cells of the 55-VNIIVViM strain is justified in our case, since it is used to develop anthrax vaccines in Russia [10]. In a recent study, it was demonstrated that live anthrax vaccines ensure the development of strong and lasting immunity to the major antigens of this pathogen [27].

Using *B. anthracis* 55-VNIIVViM (pX01+/pX02–) strain cells, three immunization schemes were tested in the experiment. The antibodies were developed best after intravenous sequential administration of the antigen every

4 days: 0.5 cm^3 with the first administration, 1.0 cm^3 with the second one and 2.0 cm^3 with the third administration at 100 million spores per 1 cm^3 concentration. After hyperimmunization the serum with an antibody level equal to 14 log_2 was obtained, which corresponds to 1:16384 titer. In similar studies, M. Caldwell et al. hyperimmunized horses 12 times with 1.0 cm^3 of spore vaccine based on *B. anthracis* Sterne strain once a month, which allowed to obtain an antibody titer of 16.25 log_2 [21]. In their experiment, C. D. Kelly et al. immunized goats with recombinant *B. anthracis* protective antigen (PA83), covalently coupled to a novel non-toxic muramyl dipeptide (NT-MDP) derivative at a dose of 100 μg. The antigen was administered on days 1, 14, 28 and 56. As a result, the authors managed to obtain the serum with an anti-PA antibody titer equal to 1:16000 [16]. Thus, the scheme we used made it possible to obtain a high level of antibodies in a shorter time.

Lyophilization is recognized as one of the best ways to preserve and store sera [15]. Therefore, two freeze-drying modes were tested to preserve the obtained serum. It was found that mode 1 produced a homogeneous creamy white serum, while mode 2 made the product excessively dry. In the process of serum freeze-drying, no protectants were used. R. Brogna et al. also demonstrated that serum lyophilization using protectants or without them does not affect the viability of immunoglobulins [28]. The antibody titer in the lyophilized serum remained at the original level for 30 months.

The developed method for serum production using rabbits hyperimmunized against anthrax has a number of advantages (low cost, ease of production, high antibody titer) and is a worthy alternative to expensive methods of hyperimmune equine sera production. The proposed technology for serum production can be used for the development of specific diagnostic products.

CONCLUSION

The studies performed allowed to elaborate the method for production of highly active hyperimmune anti-anthrax rabbit serum with an antibody titer equal to 14 log_2 was developed. The mode of the obtained serum lyophilization was optimized, which made it possible to achieve 2% residual moisture of the finished product. It was established that storage time of the lyophilized serum without loss of its original activity and physico-chemical properties is 30 months.

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Role of CFT and PCR in diagnosis of *Chlamydia psittaci* in experimentally infected rabbits

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SUMMARY

Specific antibodies against chlamydia were detected using complement fixation test and chlamydia genome was detected using polymerase chain reaction in pregnant rabbits experimentally infected with *Chlamydia psittaci*. The infected rabbits developed a fever and respiratory signs and the infection was confirmed by specific antibodies against chlamydia detected in their blood and by abnormalities in rabbit kindling. Complement fixation test of paired rabbit sera revealed an increase in the titers of specific antibodies against chlamydia, which on Day 7 post infection varied within 1:7.5; on Day 14, mean concentration was 1:40 and by Day 30 mean titer increased to 1:60. However, when pathological materials from the urogenital tract of the experimental animals were tested in polymerase chain reaction and in smear microscopy, it was impossible to confirm that there is an etiological link between chlamydia and kindling problems in experimental animals. At the same time, molecular and genetic tests of internal organs (liver) sampled from stillborn baby rabbits revealed the chlamydia genome, thus, proving chlamydia involvement into the pathological kindling. Therefore, such a retrospective method as complement fixation test with a chlamydia antigen is of high diagnostic value for lifetime chlamydia diagnosis.

Keywords: chlamydiosis, *Chlamydia psittaci*, polymerase chain reaction, rabbits, serological tests

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Значение РСК и ПЦР в диагностике экспериментальной инфекции кроликов, вызванной *Chlamydia psittaci*

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РЕЗЮМЕ

Проведена индикация специфических хламидийных антител в реакции связывания комплемента и генома хламидий методом полимеразной цепной реакции у сукрольных крольчих при экспериментальной инфекции, вызванной *Chlamydia psittaci*. Развитие инфекционного процесса у зараженных кроликов сопровождалось повышением температуры тела и появлением респираторных симптомов и было подтверждено наличием в их крови специфических хламидийных антител и патологическими родами. При исследовании парных сывороток крови кроликов в реакции связывания комплемента выявили нарастание титров специфических хламидийных антител, которые на седьмые сутки после заражения варьировались в пределах 1:7,5; на четырнадцатые сутки средняя их концентрация была равна 1:40, и к тридцатым суткам средний титр увеличился до 1:60. Однако подтвердить хламидийную этиологию неблагополучных исходов окрола экспериментально зараженных кроликов при исследовании проб патологических материалов, полученных из урогенитального тракта исследуемых животных, методом полимеразной цепной реакции и микроскопией мазков-отпечатков не удалось. При этом молекулярно-генетические исследования проб внутренних органов (печень) мертворожденных крольчат позволили выявить геном хламидий, в результате чего была подтверждена хламидийная этиология патологического исхода окрола кроликов. Следовательно, при прижизненной постановке диагноза на хламидиоз такой ретроспективный метод, как реакция связывания комплемента с хламидийным антигеном, имеет диагностическую ценность.

Ключевые слова: хламидиоз, *Chlamydia psittaci*, полимеразная цепная реакция, кролики, серологические исследования

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INTRODUCTION

Advances of molecular biology reported over the past decades have resulted in creation of new postgenomic technologies that facilitate development of methods indicating pathogen genome. These methods significantly improve laboratory diagnosis of infectious diseases, including those of animals. Polymerase chain reaction (PCR) has become one of such mainstream methods. Its high sensitivity and specificity, which, according to some authors, reach 99.9% [1–3] and surpasses all the other existing methods, together with the availability of test equipment, have contributed to a wide use of PCR for diagnosis of infectious diseases [4–7].

In the last decade of the twentieth century, genomes of almost all significant pathogens of infectious animal diseases were sequenced. Availability of nucleotide sequence databases and improvement of bioinformatic methods, along with advances in genetic engineering and artificial protein synthesis, which made it possible to obtain a polypeptide chain from amino acids, made synthesis of specific primers a routine operation.

Owing to high specificity and sensitivity of the methods based on *in vitro* nucleotide amplification, detection of the pathogen genome now plays a key role in diagnosis, pushing aside all other diagnostic methods, including retrospective diagnosis.

Wide use and availability of the method changed diagnostic algorithms, where polymerase chain reaction became the ultimate test requiring no extra confirmation and serological methods got focused only on preliminary diagnosis and monitoring with mandatory confirmation by one of direct methods, where chlamydia antigen or genome are detected in PCR[1].

However, researchers, medical and veterinary doctors, as well as laboratory workers engaged in diagnosis of infectious diseases, are familiar with such situations when diagnostic titers of specific antibodies are often detected in blood of clinically ill animals by retrospective methods, more often in complement fixation test (CFT) or enzyme-linked immunosorbent assay (ELISA) [8–11]. At the same time, titres in paired sera increased by a factor of 2 or more, which was indicative of an active infectious process, but not of a disease in the past, and PCR gave a negative result [12–15].

Thus, chlamydiosis was not confirmed, if diagnosis was based on "Methodical guidelines on laboratory diagnosis of chlamydia infections in animals", where retrospective methods are used only for preliminary diagnosis. Due to negative PCR results, many commercial breeding farms took no chlamydia eradication and prevention measures, thus leading to uncontrolled spread of chlamydia infection [16–19]. This also happened because there are simply no other diagnostic methods at disposal of veterinary laboratories, other than PCR, which detect chlamydia, antigens or DNA of chlamydia in the tested material, as prescribed in the guidelines.

Chapter 3.3.1 of the WOAH Manual of Diagnostic Tests and Vaccines for Terrestrial Animals[2] clearly recommends to use PCR and ELISA-based methods (for antigen detection) to identify a chlamydia agent. However, there are no diagnostic ELISA kits in the Russian Federation to detect it.

Therefore, PCR has become the only method used in the veterinary laboratories to diagnose chlamydia in agricultural animals. Accordingly, the aim of this research was to determine the role of CFT and PCR in diagnosis of *Chlamydia psittaci* in experimentally infected rabbits.

MATERIALS AND METHODS

For infection we used *C. psittaci* strain "250" deposited in the FSBSI "FCTRBS-ARRVI" collection of microorganisms, isolated from an aborted bovine fetus in the Kuybyshev Oblast in 1973 and cultured in developing chicken embryonated eggs with an infectious titer of $10^{-6.5}$ LD_{50}/0.3 cm^3 [20].

Five pregnant does were used for the experiment.

Sterility (non-contamination) of the pathological material for re-isolation was determined in the nutrient media: meat-peptone broth (MPB), meat-peptone agar (MPA), meat-peptone liver broth (Kitt – Tarozzi medium), Sabouraud medium.

Concentration of chlamydia-specific antibodies in blood of experimentally infected rabbits was determined with the help of CFT, using an "Antigen and sera kit for serological diagnosis of chlamydia in farm animals" (FSBSI "FCTRBS-ARRVI", Russia).

Initially, to exclude spontaneous chlamydia infection in rabbits, biomaterial samples were tested in CFT with

[1] Methodical guidelines for laboratory diagnosis of chlamydia infections in animals: approved by the Department of Veterinary Medicine on 30.06.1999 No. 13-7-2/643. Available at: https://files.stroyinf.ru/Data2/1/4293757/4293757190.htm.

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a chlamydia antigen and in PCR with a specific primer. At the next stage of the experiment, does mated with bucks. On Day 3–7 post mating, 4 does were infected with virulent chlamydia culture. Infectious material, i.e. 10% purified suspension of elementary chlamydia bodies was administered intraperitoneally in a volume of 1.0 cm^3. One rabbit served as a control.

On Day 23–30 post infection, the effect of kindling was assessed. The kits from the does were visually examined, height and weight measured and diagnostic tests for chlamydia made to confirm the etiological link with the pathogen in case of reproductive disorders in the infected animals.

Microscopic tests included sampling, preparation of smears, carbol-fuchsin staining (Stamp's method) and micro-slide examination under the immersion system of a light microscope at a magnification of 100×.

The does were sampled in accordance with the PCR sampling rules. Urogenital probes were used to sample vaginal mucosa of the infected animals, after that the samples were put into sterile tubes containing transport medium. Samples for microscopy were also taken with urogenital probes and applied onto slides to prepare smears.

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

PCR test was used for molecular and genetic purposes. Chlamydia DNA was detected in samples from the infected animals using PCR-based "CHLA-KOM" test kit for chlamydia detection in animals and birds (FBIS "Central Research Institute of Epidemiology" of Rospotrebnadzor, Russia), developed to detect DNA of *Chlamydiaceae* family microorganisms in biological material.

DNA was extracted from the tested material using a set of reagents "DNA-sorb-B" (FBIS "Central Research Institute of Epidemiology" of Rospotrebnadzor, Russia). Amplification products were detected in agarose gel electrophoresis using the "EF" kit for preparation of agarose gel (FBIS "Central Research Institute of Epidemiology" of Rospotrebnadzor, Russia). The results were read judging by the presence or absence of a specific band of amplified DNA in the electrophoregram.

Chlamydia was isolated from pathological and clinical material taken from developing 6-day-old chicken embryoned eggs. For this purpose, 20% suspensions were prepared from the liver of stillborn rabbits (2 fetuses from each doe), which were subsequently administered into the yolk sac of developing chicken embryonated eggs. Embryos that died earlier than Day 4 post infection were culled (as nonspecific). Yolk membranes were sampled from the embryos that died between Day 4 and Day 14 post infection, smears were prepared from them, stained using modified Stamp's method and examined under an immersion light microscope in order to identify elementary chlamydia bodies in the form of pink dots on a green mounting media.

RESULTS AND DISCUSSION

The experiment was conducted in the Department of Virology of the FSBSI "FCTRBS-ARRVI".

At the first stage, general health status of the experimental animals was assessed.

Observation of animals before insemination and on Days 3–7 after insemination, as well as before infection revealed that the rabbit health parameters were within the physiological norm, as confirmed by the body temperature (38.5–39.5 °C) and animal behavior.

No specific antibodies were detected in sera samples tested in CFT with a chlamydia antigen. No chlamydia genome was detected in PCR-tested clinical material from these animals.

Prior to the experiment, 4 bucks were clinically examined; then used for mating with experimental does and subjected to similar laboratory tests for spontaneous chlamydia infection. The results were also negative.

As thermometry showed, infection with a virulent chlamydia strain caused a short increase in body temperature in all 4 experimental rabbits, which began on Day 2 after infection and lasted until Day 6, when the average body temperature in the group was equal to 39.62 °C. In the following days, the temperature began to stabilize. In addition, the infection affected the overall health status of the rabbits. Starting from Day 3, the animals lose appetite, look lethargic and unkempt. On Day 5, three infected rabbits (Nos. 1, 2 and 3) developed a cough.

Assessment of kindling post infection with virulent chlamydia is given in Table 1.

Table 1
Effect of experimental infection with *C. psittaci* "250" on pregnancy in rabbits

Animal number	Group	Effect on pregnancy	Kits born in total	Kits died		Kits survived	
				number	%	number	%
1	Experimental	s/b	6	6	100	0	0
2		s/b	5	5	100	0	0
3		kindling, s/b	6	2	33	4	67
4		s/b	5	5	100	0	0
Total for the group			22	18	82	4	18
5	Control	kindling	6	0	0	6	100

"s/b" – stillbirth.

Stillbirth cases were reported in three infected animals. Only four out of six kits from Doe No. 3 survived. The survival rate in the group was 18%. The control doe gave birth to 6 healthy kits.

The next stage was to study humoral immunity dynamics in rabbits. The results of serological tests of sera from experimental and control animals are given in Table 2.

It was found that, prior to infection no antibodies to chlamydia antigen were detected in blood of all rabbits. Consequently, they lacked specific anti-chlamydial immunity, which was important for purity of the experiment. On Day 7 post infection, complement-fixing antibodies were detected in sera from experimental rabbits; mean titer was 1:7.5. In the future, there was an increase in their concentration. Therefore, on Day 14, mean titer was equal to 1:40 and by Day 30, it increased to 1:60. An increase in the antibody level in the paired serum samples from the infected rabbits suggests development of an infectious process of chlamydial etiology. No complement-fixing antibodies were detected in blood of the control animals during the whole experiment.

Mucus scrapes from vagina of infected animals were taken together with the samples of internal organs from the stillborn and normal kits (liver) for microscopic, molecular and genetic analyses aimed at detection of chlamydia elementary bodies and DNA on Day 30 post infection, after kindling in all does.

Table 3 summarizes data on fetuses and clinical materials tested after kindling, as well as on chlamydia re-isolation from pathological materials.

Microscopic tests showed no chlamydia in vaginal smears of the infected does. Microscopic tests showed elementary bodies of chlamydia in smears prepared from the internal organs of stillborn kits. PCR revealed no DNA chlamydia in samples of vaginal mucosa. PCR revealed *C. psittaci* genetic material in liver samples from stillborn kits. Similar tests of the pathological material taken from the control rabbit gave negative results.

To identify the pathogen and confirm chlamydial etiology of the abnormal kindling observed in does, the pathogen was re-isolated from samples of internal organs (liver) of the stillborn fetuses with positive PCR results in developing 6-day-old chicken embryonated eggs, followed by a smear microscopy to detect elementary bodies of chlamydia. Chlamydia were already isolated in the first passage. Specific embryonic death was reported on Days 4–8 after infection with a 20% suspension of pathological materials. In addition to confirming the chlamydial etiology of stillbirths in rabbits, it also suggested a high virulence of chlamydia isolates re-isolated from rabbits. Administration of pathological materials from the liver of control rabbit fetuses into the yolk sac of developing chicken embryonated eggs showed a negative result.

Analyzing the data obtained during the experiment, it can be concluded that the use of PCR alone gives an incomplete picture for the chlamydia lifetime diagnosis. It is advisable to use retrospective methods, such as CFT with a chlamydia antigen, which makes it possible to identify chlamydia specific antibodies. An increase in the antibody titre in the animal blood may suggest an active infectious process and may be used as a basis for a diagnosis.

Table 2
Level of complement-fixing antibodies in blood of infected rabbits

Animal number	Group	Chlamydia antibody titer			
		before infection	Day 7	Day 14	Day 30
1	Experimental	–	1:10	1:20	1:40
2		–	1:5	1:20	1:40
3		–	1:10	1:80	1:80
4		–	1:5	1:40	1:80
Mean titre in group		–	1:7.5	1:40	1:60
5	Control	–	–	–	–

Table 3
Re-isolation of *C. psittaci* "250" from clinical and pathological material from infected rabbits after kindling

Animal number	Group	Test material	Microscopy results	Re-isolation of chlamydia in chicken embryonated eggs	PCR results
1	Experimental	mucus scrape from vagina	–	not tested	–
		fetal liver	+	+	+
2		mucus scrape from vagina	–	not tested	–
		fetal liver	+	+	+
3		mucus scrape from vagina	–	not tested	–
		fetal liver	+	+	+
4		mucus scrape from vagina	–	not tested	–
		fetal liver	+	+	+
5	Control	mucus scrape from vagina	–	not tested	–
		fetal liver	–	–	–

"–" – negative test results;
"+" – positive test results.

CONCLUSION

The conducted experiments showed that infection of pregnant does with virulent *Chlamydia psittaci* strain "250" resulted in an active infectious process, characterized by a fever, respiratory signs and deterioration in the general condition of the tested animals. In addition, all experimental does had abnormal kindling. Only 4 out of 22 kits survived. The offspring survival rate was 18%. Only serological tests (CFT) made it possible to confirm chlamydial etiology of the pathological process in the infected animals. Imprint smear microscopy and PCR of urogenital tract scrapes from the infected animals gave negative results. However, the chlamydial etiology of abnormal kindling was confirmed by PCR, used to test internal organs from fetuses of the infected animals and by chlamydia re-isolation in 6-day-old chicken embryonated eggs.

Thus, the use of PCR alone does not give a complete picture for chlamydia lifetime diagnosis in animals. Consequently, it is advisable to use not only molecular, but also retrospective methods, such as CFT with a chlamydia antigen, which helps to identify specific chlamydia antibodies, and the growing antibody titre can serve as a basis for the diagnosis.

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Metabolism features and milk microbiota of cows with mastitis in the Amur Oblast

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SUMMARY

The effective management practices of dairy farming are inextricably linked with the production of high quality milk, while the mastitis is one of the causes of reductions in milk yields and quality. The aim of the work was to study the microbiological profile of milk, the sensitivity of isolated microorganisms to antibiotics and metabolism features of cows with mastitis in the Amur Oblast. The following microorganisms were identified in the milk samples from cows with mastitis: *Staphylococcus epidermidis* (34.69%); microbial associations: *Streptococcus agalactiae* + *Escherichia coli* (32.65%) and *Staphylococcus saprophyticus* + *Streptococcus agalactiae* + *Escherichia coli* (30.61%); *Staphylococcus haemolyticus* (2.05%). Microorganisms isolated from the milk of mastitis-affected cows were susceptible to the following antimicrobials: *Escherichia coli* to cefotaxime (28.00 ± 2.00 mm) and ceftriaxone (27.50 ± 0.35 mm); *Staphylococcus epidermidis*, *Staphylococcus haemolyticus*, *Staphylococcus saprophyticus* to cefotaxime (35.50 ± 0.18 mm) and amoxicillin (35.10 ± 0.35 mm); *Streptococcus agalactiae* to tetracycline (27.60 ± 1.17 mm) and gentamicin (26.40 ± 0.99 mm). Metabolic disorders were observed in cows with mastitis. The albumin-globulin ratio was reduced (0.41), which is typical for various inflammatory processes; and a low albumin levels (29.00 ± 0.89%) suggested a decreased protein synthesis in hepatocytes. A moderate increase in gamma globulins (47.60 ± 1.05%) was associated with stimulation of the phagocytic mononuclear system. Water and mineral metabolism disorders were confirmed by low levels of calcium (1.80 ± 0.03 mmol/L) and magnesium (0.70 ± 0.02 mmol/L), which is a sign of many pathological conditions, and is associated with heavy lactation. Calcium-phosphorus ratio was reduced (0.82). Hematological indicators suggested hypochromic anemia (hemoglobin level – 100.60 ± 1.28 g/L, globular value – 0.60 ± 0.01). The leukogram was indicative of lymphocytopenia (36.90 ± 2.60%) and neutrophilia (rod-shaped neutrophils – 1.80 ± 0.13%, segmented neutrophils – 51.80 ± 2.51%).

Keywords: Amur Oblast, mastitis, cattle, mammary gland, opportunistic microorganisms, microbiological test, antibiotics, hematological tests

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Метаболические особенности и микрофлора молока при маститах у коров Амурской области

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РЕЗЮМЕ

Эффективное ведение молочного скотоводства неразрывно связано с получением молока высокого санитарного качества, одной из причин снижения производства которого являются маститы. Цель работы заключалась в исследовании микробиологического профиля молока, определении чувствительности выделенных микроорганизмов к антибиотикам и изучении метаболических особенностей при маститах у коров Амурской области. В животноводческих хозяйствах Амурской области в пробах молока от больных маститом коров в 34,69% случаев выявляли *Staphylococcus epidermidis*; в 32,65 и 30,61% проб обнаруживали ассоциации микроорганизмов: *Streptococcus agalactiae* + *Escherichia coli* и *Staphylococcus saprophyticus* + *Streptococcus agalactiae* + *Escherichia coli* соответственно; доля *Staphylococcus haemolyticus* составила 2,05%. Выделенные из молока от больных маститом коров *Escherichia coli* были чувствительны к цефотаксиму (28,00 ± 2,00 мм) и цефтриаксону (27,50 ± 0,35 мм); *Staphylococcus epidermidis*, *Staphylococcus haemolyticus*, *Staphylococcus saprophyticus* – к цефотаксиму (35,50 ± 0,18 мм) и амоксициллину (35,1 ± 0,35 мм); *Streptococcus agalactiae* – к тетрациклину (27,60 ± 1,17 мм) и гентамицину (26,40 ± 0,99 мм). У больных маститом коров отмечали нарушения метаболизма. Был снижен альбумин-глобулиновый коэффициент (0,41), что характерно для воспалительных процессов различной локализации, а низкий уровень альбуминов (29,00 ± 0,89%) свидетельствовал о снижении протеинсинтетической функции гепатоцитов. Умеренное увеличение гамма-глобулинов (47,60 ± 1,05%) было связано с раздражением системы фагоцитирующих мононуклеаров. Нарушения водно-минерального обмена характеризовались низким уровнем кальция (1,80 ± 0,03 ммоль/л) и магния (0,70 ± 0,02 ммоль/л), что отмечается при многих патологических состояниях, а также при усиленной лактации. Соотношение кальция и фосфора было

снижено (0,82). Гематологические показатели свидетельствовали о гипохромной анемии (уровень гемоглобина – 100,60 ± 1,28 г/л, цветового показателя – 0,60 ± 0,01). Лейкограмма указывала на лимфоцитопению (36,90 ± 2,60%) и нейтрофилию (палочкоядерных нейтрофилов – 1,80 ± 0,13%, сегментоядерных нейтрофилов – 51,80 ± 2,51%).

Ключевые слова: Амурская область, мастит, крупный рогатый скот, молочная железа, условно-патогенные микроорганизмы, микробиологическое исследование, антибиотики, гематологические исследования

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INTRODUCTION

The effective management practices of dairy farming are inextricably linked with the production of high quality milk, while the mastitis is one of the causes of reductions in milk yields and quality [1–3]. The disease can develop during all periods of the cow's udder functionality, but most often during lactation (36%) and drying off (23%). The critical periods include a dry period (16%) and a period immediately after calving (25%) [4].

Microorganisms can enter the mammary gland using different ways: galactogenic (most often), hematogenic and lymphogenic ways [5]. The galactogenic infection of the mammary gland with microorganisms occurs in high yielding dairy cows suffering from metabolic disorders and decreased immunity.

Feeding and maintenance conditions contribute to the bacterial manifestation of pathogenic and opportunistic microorganisms most often detected in case of mastitis: *Staphylococcus aureus*, *Staphylococcus xylosus*, *Staphylococcus epidermidis*, *Streptococcus dysgalactiae*, *Streptococcus uberis*, *Escherichia coli*, *Pseudomonas aeruginosa* [1, 6–11].

Different generations of antibiotics are used to treat mastitis in cattle, but their misuse can lead to antimicrobial resistance [1].

Metabolomic analysis of cows with mastitis, hematological tests, bacteriological test of milk and antimicrobial susceptibility testing will contribute to correct and timely determination of treatment strategy.

The aim of the work is to study the microbiological profile of milk, the susceptibility of microorganisms to antibiotics and metabolic features in cows with mastitis in the Amur Oblast.

MATERIALS AND METHODS

The experiments were performed in livestock farms of the Amur Oblast using tie-stall housing. 84 milk samples from 21 Holstein cross-bred cows were tested during lactation (2–4 lactations).

Samples of alveolar milk were taken from each quarter of the udder using sterile procedure for testing. The preliminary diagnosis of mastitis and differentiation by types of mastitis was made using the rapid test-kit "Masttest" (LLC NPP "Agrofarm", Russia).

The bacteriological test was performed in accordance with the "Manual on the diagnosis, treatment and prevention of mastitis in cows"[1], "Recommended practices of bacteriological test of cow milk and udder secretion"[2], GOST 32901-2014 "Milk and dairy products. Methods of microbiological testing"[3].

Kessler and Endo media were used to isolate and identify *E. coli*. Smears from the isolated colonies were Gram stained. When staphylococci were detected in smears, the isolated culture was checked for catalase activity (gas production in response to hydrogen peroxide). To isolate streptococci from milk, a Kartashova solid medium was used. Streptococci were differentiated from staphylococci by the presence of catalase activity.

The susceptibility of bacteria to the following antimicrobials was tested on solid nutrient media using disc diffusion[4] susceptibility test: ampicillin, amoxicillin, novobiocin, neomycin, benzylpenicillin, cefotaxime, kanamycin, ceftriaxone, doxycycline, polymyxin, gentamicin, tetracycline.

Blood was taken from the caudal vein and stabilized with heparin for hematological testing. Metabolic panel serum tests were performed using StatFax 1904+R photometer (Awareness Technology, Inc., USA) and a set of reagents manufactured by company "Vital Development Corporation" (Russia). Hematological tests were performed according to generally accepted methods.

All experiments were carried out in strict accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS No. 123).

Statistical processing of the results was performed by standard methods using the MS Excel software package.

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RESULTS AND DISCUSSION

58.33% samples showed a clot of various densities following the reaction of "Masttest" surfactant (sulfonol) with the somatic cell nuclear DNA, which was indicative of the udder inflammation. The interactions of the milk mixture with the diagnosticum differed and gave the following results: 36.90% – inconclusive; 5.95% (+), 15.48% (++) – positive; 41.67% samples showed no reaction (negative result).

The udder examination revealed that most often (28.57%) the inflammatory process was localized in the posterior lobes of the udder. Lesions of the anterior left lobes (24.49%) came second, the affected anterior right lobes (18.37%) came third.

The greatest lesions (++ according to "Masttest" results) were found in the udder posterior lobes: 57.14% – in the left and 35.71% – in the right lobe.

The CFU/g value in the affected udder lobes [$(34.70 \pm 2.46) \times 10^5$, $n = 49$] differed significantly ($p < 0.001$) from the CFU/g value in the healthy udder lobes [$(15.40 \pm 0.86) \times 10^5$, $n = 35$].

Milk samples from cows with mastitis revealed: 1) *Staphylococcus epidermidis* (34.69%); 2) microbial associations *Streptococcus agalactiae* + *Escherichia coli* (32.65%) and *Staphylococcus saprophyticus* + *Streptococcus agalactiae* + *Escherichia coli* (30.61%); 3) *Staphylococcus haemolyticus* (2.05%).

The antimicrobial susceptibility test showed the highest susceptibility to the following antimicrobials ($n = 24$): *Escherichia coli* to cefotaxime (28.00 ± 2.00 mm, $p < 0.001$) and ceftriaxone (27.50 ± 0.35 mm, $p < 0.001$); *Staphylococcus epidermidis*, *Staphylococcus haemolyticus*, *Staphylococcus saprophyticus* to cefotaxime (35.50 ± 0.18 mm, $p < 0.001$) and amoxicillin (35.10 ± 0.35 mm, $p < 0.001$); *Streptococcus agalactiae* to tetracycline (27.60 ± 1.17 mm, $p < 0.001$) and gentamicin (26.40 ± 0.99 mm, $p < 0.001$).

Metabolic panel blood test showed increased levels of total protein (94.70 ± 0.75 g/L, $p < 0.001$) and gamma globulins (47.60 ± 1.05%, $p < 0.001$); decreased albumins (29.00 ± 0.89%, $p < 0.001$) and alpha globulins (10.60 ± 0.72%, $p < 0.001$). The levels of beta globulin (12.80 ± 0.69%), urea (4.40 ± 0.10 mmol/L) and creatinine (67.10 ± 0.87 μmol/L) were within physiological limits. The albumin-globulin ratio was reduced to 0.41 (limits 1.2–1.8).

Bilirubin levels (8.50 ± 0.44 μmol/L, $p < 0.001$) exceeded the normal limits, the aminotransferase activity (ALT and AST, 21.50 ± 0.61 and 97.40 ± 8.48 units/L respectively) was within physiological limits, and glucose concentration (1.00 ± 0.02 mmol/L, $p < 0.001$) was low.

Water and electrolyte metabolism analysis showed that against the normal values of phosphorus (2.20 ± 0.04 mmol/L) and potassium (4.50 ± 0.06 mmol/L), the levels of calcium (1.80 ± 0.03 mmol/L, $p < 0.001$) and magnesium (0.70 ± 0.02 mmol/L, $p < 0.001$) were decreased. The calcium and phosphorus ratio was 0.82.

Hematological tests revealed increased red blood cells [$(8.80 \pm 0.14) \times 10^{12}$/L, $p < 0.001$], low hemoglobin levels (100.60 ± 1.28 g/L, $p < 0.001$) and color index (0.60 ± 0.01, $p < 0.001$), which suggested anemia. White blood cells [$(7.70 \pm 0.40) \times 10^9$/L, $p < 0.001$] were within physiological limits, but the leukogram showed decreased lymphocytes (36.90 ± 2.60%, $p < 0.001$) and rod-shaped neutrophils (1.80 ± 0.13%, $p < 0.001$), as well as higher segmented neutrophil levels (51.80 ± 2.51%, $p < 0.001$).

As a result of the tests performed, it was found that the following microorganisms are identified in milk of cows with mastitis: *Staphylococcus haemolyticus*, *Staphylococcus epidermidis*, *Staphylococcus saprophyticus*, *Streptococcus agalactiae* and *Escherichia coli*, which is consistent with the publications of other researchers [12–16].

Milk of cows with mastitis contained one representative of opportunistic microbiota (*Staphylococcus epidermidis* – 34.69% and *Staphylococcus haemolyticus* – 2.05%) and also microbial associations (*Streptococcus agalactiae* + *Escherichia coli* – 32.65% and *Staphylococcus saprophyticus* + *Streptococcus agalactiae* + *Escherichia coli* – 30.61%).

During their lifetime the isolated staphylococci and streptococci, depending on the type of secreted toxins, damage the membranes of erythrocytes, leukocytes, hepatocytes, cardiomyocytes, connective tissue cells; they suppress phagocytosis and dissolve torus demarcationis fibrin in the process of inflammation; disturb osmotic pressure and facilitate cell lysis; separately and together with enterobacteria they can cause exogenous infections [17].

The susceptibility testing of microorganisms isolated from milk of cows with mastitis revealed that the largest zones of inhibition of enterobacteria and staphylococci were formed in response to cefotaxime, and streptococci were sensitive to tetracycline and gentamicin. Therefore, in case of mammary gland inflammation caused by microbial associations, it is necessary to use several antimicrobials.

The analysis of protein metabolism in cows with mastitis showed lower albumin-globulin ratio (0.41), which is consistent with various inflammatory processes, and a low level of albumins (29.00 ± 0.89%) suggested a decrease in the protein synthesis in hepatocytes. A moderate increase in gamma globulins (47.60 ± 1.05%) was associated with stimulation of the phagocytic mononuclear system [18].

Disorders in water and mineral metabolism were characterized by low levels of calcium and magnesium, which is characteristic for many pathological conditions, as well as for heavy lactation. Calcium-phosphorus ratio, equal to 0.82, is typical for cows in dry period. For lactating cows, this value should be 1.2–1.8 [18].

General blood test showed hypochromic anemia, lymphocytopenia and neutrophilosis in animals, which is typical for inflammatory processes [19].

CONCLUSION

In livestock farms of the Amur Oblast, *Staphylococcus epidermidis* was detected in 34.69% of milk samples from cows with mastitis; microbial associations were revealed in 32.65 and 30.61% of samples: *Streptococcus agalactiae* + *Escherichia coli* and *Staphylococcus saprophyticus* + *Streptococcus agalactiae* + *Escherichia coli*, respectively; *Staphylococcus haemolyticus* was detected in 2.05% of samples.

Escherichia coli isolated from milk of mastitis-affected cows were sensitive to cefotaxime and ceftriaxone; *Staphylococcus epidermidis*, *Staphylococcus haemolyticus*, *Staphylococcus saprophyticus* to cefotaxime and amoxicillin; *Streptococcus agalactiae* to tetracycline and gentamicin.

Metabolic disorders were observed in cows with mastitis. The albumin-globulin ratio was reduced, which suggests the development of inflammatory processes, and low albumin levels were indicative of impaired protein synthesis in hepatocytes. A moderate increase in gamma

globulins was associated with stimulation of the phagocytic mononuclear system. Disorders in water and mineral metabolism were characterized by low levels of calcium and magnesium, which is characteristic for many pathological conditions, as well as for heavy lactation. Calcium-phosphorus ratio was low.

Hematological indicators suggested hypochromic anemia, and the leukogram was indicative of lymphocytopenia and neutrophilia.

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Epizootic situation for infectious bovine rhinotracheitis in the Karaganda Oblast, the Republic of Kazakhstan, in 2021–2022

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SUMMARY

A comprehensive serological monitoring is currently underway in the Republic of Kazakhstan to detect the circulation of the infectious bovine rhinotracheitis pathogen. To conduct a full-fledged and resultful study, the principles of sampling size representativeness provision and mathematical calculations were observed. The sampling size of the total number of epizootological units included mainly the raions and settlements in which (or near which) infectious bovine rhinotracheitis cases had been previously recorded. The sampling size of livestock population included in the study was determined in accordance with the recommendations of the World Organization for Animal Health. Thus, the study covered 7 (out of 13) raions of the Karaganda Oblast in 2021 and 2022. The other 6 raions and cities of regional significance will be included in the research in 2023. The paper presents the results of infectious bovine rhinotracheitis retrospective analysis and own studies conducted in 2021–2022. Statistical analysis and graphical visualization of investigation results were performed using Statistica, Excel, and QGIS programs. It was established that the epizootic situation for this disease was unfavourable in the Karaganda Oblast in 2021–2022. The data and results of serological studies presented by the Veterinary Control and Surveillance Committee of the Ministry of Agriculture of the Republic of Kazakhstan confirm the circulation of the infectious bovine rhinotracheitis virus in the following raions of the Karaganda Oblast: Abaisky, Aktogaisky, Bukhar-Zhyrausky, Karkaralinsky, Nurinsky and Osakarovsky.

Keywords: epizootological monitoring, infectious bovine rhinotracheitis, epizootic situation, infection outbreaks

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Эпизоотическая обстановка по инфекционному ринотрахеиту крупного рогатого скота в Карагандинской области Республики Казахстан в 2021–2022 гг.

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РЕЗЮМЕ

В настоящее время в Республике Казахстан проводится полноценный серологический мониторинг на предмет обнаружения циркуляции возбудителя инфекционного ринотрахеита крупного рогатого скота. Для проведения полноценного и показательного исследования соблюдались принципы обеспечения репрезентативности и математического расчета выборки. При формировании выборки среди общего количества эпизоотологических единиц большее внимание уделялось районам и населенным пунктам, в которых (или вблизи которых) ранее регистрировали случаи инфекционного ринотрахеита крупного рогатого скота. Выборка среди поголовья, вошедшего в область исследования, осуществлялась в соответствии с рекомендациями Всемирной организации здравоохранения животных. Таким образом, в область исследования в 2021 и 2022 гг. вошли 7 районов Карагандинской области из 13 возможных. Оставшиеся 6 районов и городов областного значения будут исследованы в 2023 г. В статье представлены результаты ретроспективного анализа по инфекционному ринотрахеиту крупного рогатого скота и собственных исследований, выполненных в 2021–2022 гг. С помощью программ Statistica, Excel, QGIS проведена статистическая и графическая обработка результатов исследования. При изучении эпизоотической обстановки установили, что Карагандинская область в 2021–2022 гг. была неблагополучна по заболеванию. Представленные Комитетом ветеринарного контроля и надзора Министерства сельского хозяйства Республики Казахстан данные и результаты серологических исследований подтверждают факт циркуляции возбудителя инфекционного ринотрахеита крупного рогатого скота в следующих районах Карагандинской области: Абайском, Актогайском, Бухар-Жырауском, Каркаралинском, Нуринском и Осакаровском.

Ключевые слова: эпизоотологический мониторинг, инфекционный ринотрахеит крупного рогатого скота, эпизоотическая ситуация, очаги инфекции

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INTRODUCTION

Infectious bovine rhinotracheitis (IBR) is a contagious disease that may be persistent and which is characterized by lesions in the respiratory and reproductive organ systems of animals. One of the most distinctive signs of this disease is hyperemia of the mucous membranes of the muzzle and nares, also called "red nose". Such a typical manifestation of IBR is noted in most cases in young animals under 12 months [1].

The disease pathogen is a DNA-genomic bovine alphaherpesvirus 1 belonging to the *Orthoherpesviridae* family. Two subtypes of the virus have been identified to date: 1.1 and 1.2. Type 1 virus affects the respiratory organs, rarely the reproductive organs, type 2 virus causes genital infection. To date, the existence of another subtype of bovine alphaherpesvirus has been proven, but some of its features give grounds for attributing the pathogen to another type [2]. Like most respiratory pathogens, the IBR virus is shed into the external environment mainly through the organs of the respiratory system [3].

The IBR causative agent is quite resistant to environmental conditions. It remains virulent at 22 °C for 45 days, at 4 °C – for up to 7 months. Deep freezing does not affect the virus activity, the infectious titer is stable for 7–9 months. When heated above 56 °C, it dies within 7–20 minutes. The virus is inactivated when treated with chlorine, 2% formalin or sodium hydroxide solutions [2, 4].

The disease may occur in various forms: respiratory form with the upper respiratory tract lesions and genital form with lesions in external genitalia. In addition, IBR is characterized by abortions and conjunctivitis. Systemic infection is often observed in cattle infected in the late stages of pregnancy *in utero* or in neonatal calves [5, 6].

The sources of infection are diseased animals and latent virus carriers. Other than asymptomatic carriers, breeding bulls pose the greatest risk of infectious rhinotracheitis, since the pathogen is capable of transmitting from animal to animal through semen [1, 7–10].

Many researchers have noted the possibility of simultaneous presence of IBR virus and antibodies to it. Thus, the virus could be isolated from the tonsils and lymph nodes of animals with high antibody titers [1, 11–13].

IBR can be treated symptomatically, infected animals shall be isolated from the rest of the herd, a specific serum against this disease may be used.

IBR control is based on vaccination [14, 15], which is considered the most effective way of prevention. Currently, attempts are being made to use subunit, recombinant or split vaccines to reduce the spread or eradicate IBR in different countries [8, 16–23]. Along with imported medicinal products, the inactivated adsorbed vaccines (OOO "NPO Narvac") and adsorbed and emulsion vaccines (FGBI "ARRIAH") are administered in the Russian Federation. Such live vaccines as Bovi-scield Gold FP5 L5, INFORCE 3® (Zoetis Inc., USA) and others are quite popular in the European countries [24, 25].

The IBR epizootic situation in the Republic of Kazakhstan remains tense. Many regions of the country are infected. The mean seroprevalence ranges from 65 to 87% for unvaccinated animals, which clearly demonstrates the circulation of the pathogen among cattle in the Republic.

Infectious rhinotracheitis is officially included in the list of highly dangerous bovine diseases along with foot-and-mouth disease, lumpy skin disease and anthrax. The disease-induced economic losses include animal culling, decreased productivity and emergency slaughter [26–30]. No comprehensive epizootological monitoring of IBR is currently conducted in the Republic of Kazakhstan. Laboratory diagnosis is carried out only in the areas where outbreaks were previously reported. At the same time, only 5–10 samples are collected from livestock for testing, which is not indicative due to the small sampling size. In view of the above, it is an urgent and necessary task to study the disease and conduct the IBR monitoring. Rationale for the sampling size of epizootological units and susceptible livestock will allow realistic assessment of the disease situation and will make it possible to effectively implement epizootological control measures and plan the new ones.

The purpose of this study was to conduct a retrospective analysis and evaluate the current (2021–2022) epizootic situation for IBR in the Karaganda Oblast based on the sampling of epizootological units and target animals in them.

MATERIALS AND METHODS

In order to determine the IBR epizootological status of the Karaganda Oblast, it was necessary to collect and summarize the epizootological data available at present, evaluate the measures used in the disease outbreaks, as well as highlight the weak points of the veterinary measures aimed at preventing the introduction and spread of the pathogen in the country. For that, the relevant requests were sent to the Veterinary Surveillance Committee of the Ministry of Agriculture of the Republic of Kazakhstan for providing data on IBR cases reported in 2021–2022.

To assess the IBR current situation in the Karaganda Oblast, a sample of epizootological units and susceptible livestock was formed. In order to ensure the sample reliability, we followed the recommendations of the World Organisation for Animal Health (WOAH) and the "Guidelines on formation of an epizootological unit (EU) and a sample based on all epizootological units in order to conduct examination and determine the number of animals required for subsequent studies for establishment of the epizootological status of herds, economic entities and zoo habitats", developed and approved by LLP "KazNIVI" based on the WOAH provisions and recommendations.

In this study we used enzyme-linked immunosorbent assay (ELISA), a serological method of primary importance in IBR diagnosis. In addition, according to the WOAH recommendations, ELISA is determined as the most acceptable method for proving the absence or presence of the pathogen circulation in total population[1].

Other factors for selecting the diagnostic method were the relative cheapness and rapidness in comparison with the polymerase chain reaction, which is 1.5–2.0 times more expensive than ELISA, as well as with the test for pathogen isolation and differentiation in cell culture, which are time-consuming.

IDEXX IBR gB X3 Ab Test (infectious bovine rhinotracheitis) commercial diagnostic kit for IBR antibody detection (manufacturer: IDEXX Laboratories, Inc., USA) was used for testing.

RESULTS AND DISCUSSION

The Karaganda Oblast as the administrative unit of the Republic of Kazakhstan was selected for the study due to its status of a continuously infected region.

In the period from 2010 to 2012 the Karaganda Oblast was free from IBR. No pathogen circulation was detected during planned monitoring. However, in 2013 the IBR suspect cases were registered in the Subjects of the Kyzylaraisky (Aktogaisky Raion) and Zharaspaisky (Nurinsky Raion) rural districts (based on citizens' reports). Tests of samples collected from animals with the disease clinical signs demonstrated no laboratory confirmation of the diagnosis.

In 2014 no IBR cases were recorded in the Karaganda Oblast.

In 2015 the reports on IBR suspect cases in Jezkazgan were submitted again. Restrictive measures were imposed in the settlement. Same as in 2013, these cases were managed in the laboratory, and negative results were obtained. It should be noted that the spread of the pathogen that caused a respiratory animal disease in Jezkazgan was prevented.

New reports of citizens on occurrence of respiratory disease symptoms in cattle were received in 2016 from the Amanzholovsky rural district, while other settlements of the Karkaralinsky Raion were not affected. Restrictive measures introduced on the territory of this settlement made it possible to resolve the recorded outbreaks of a respiratory disease. It should be added that testing of samples from IBR suspect animals did not confirm the diagnosis. Other raions of the Karaganda Oblast remained disease-free in 2016.

In 2017 an IBR suspect case was recorded in Karaganda. According to the decision of the local executive body and the chief veterinary officer of the Karaganda Oblast, restrictive measures were administered in the infected locality, which made it possible to resolve the epizootic outbreak and prevent the spread of respiratory infection. Testing of biomaterial samples from IBR suspects in the laboratory did not demonstrate the diagnosis confirmation.

In 2018 there were no reports on IBR suspect cases in the region.

In 2019 several citizen reports on IBR suspects in several farms of the Yntalinsky rural district of the Karkaralinsky Raion were submitted. Restrictive measures were conducted in this locality in accordance with the decision of the local executive authority and the chief veterinary officer of the Karaganda Oblast. However, it was not possible to confirm the diagnosis by laboratory testing of samples from IBR suspect animals.

Such a situation may indicate an asymptomatic, or latent, form of infection in cattle in the region. Besides, the IBR pathogen persistence and the infectious process development in cattle in the herd are closely related to decreased immunity and, as a consequence, occurrence of the virus-susceptible animals.

The results of epizootological monitoring showed that in 2020 new IBR suspect cases were registered in the Karaganda Oblast in Mibulaksky (Ulytausky Raion), Akbastausky (Abaisky Raion) and Kulanotpessky (Nurinsky Raion) rural districts. Restrictive measures were implemented in these settlements.

According to the data available, 7 IBR suspect cases were registered in the Karaganda Oblast in 2021. The first one was detected on 6 January 2021 in Tasaralsky rural district of Aktogaisky Raion. However, the disease was not confirmed by laboratory tests.

A new report on IBR suspicion was received from the Ulytausky Raion on 12 March 2021. Despite the negative laboratory test results, 5 more reports on disease symptomatic manifestations were registered until November 2021 not only in the Ulytausky and Aktogaisky, but also in the Shetsky and Nurinsky Raions. In the Shetsky Raion 2,600 animals were vaccinated and the premises (4,325 m^2) where diseased animals were kept were disinfected based on the recommendations.

Taking into account the IBR current epizootic situation in 2021 the Kazakh Scientific Research Veterinary Institute conducted additional monitoring studies to confirm or rule out the possible spread of the disease pathogen. Thus, 3 raions of the Karaganda Oblast (Abaisky, Osakarovsky and Nurinsky) were subjected to study.

[1] Infectious bovine rhinotracheitis/infectious pustular vulvovaginitis. *In: WOAH. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. Chapter 3.4.11.* Available at: https://www.woah.org/fileadmin/Home/eng/Health_standards/tahm/3.04.11_IBR_IPV.pdf.

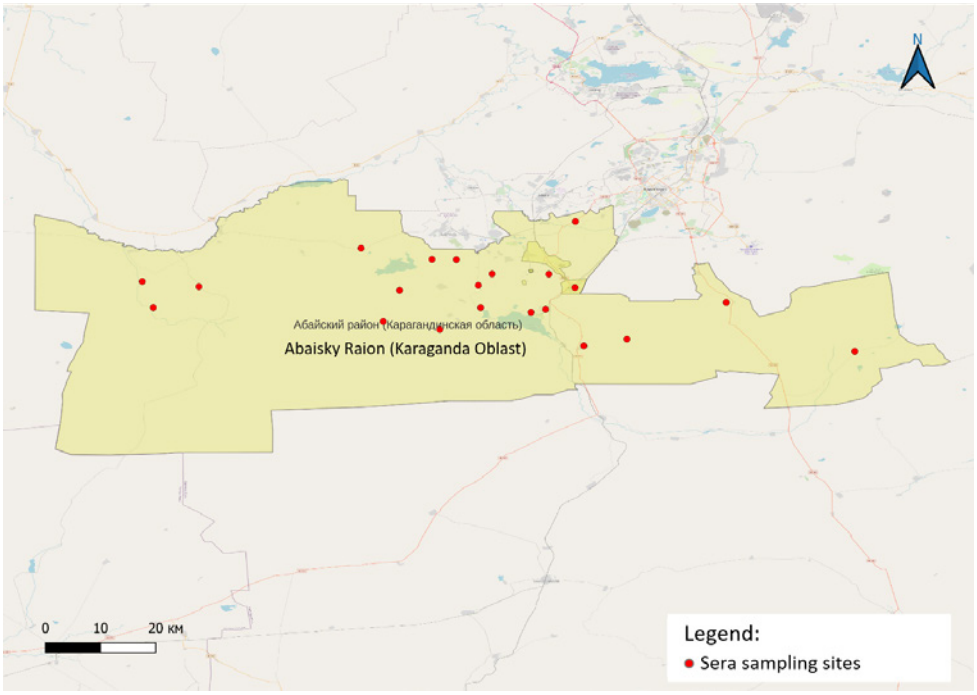

Fig. 1. Sampling sites for blood collection in the Abaisky Raion, the Karaganda Oblast (2021)

The location of epizootological units (EU) used for sampling in the Abaisky Raion is shown in Figure 1. Here, the sampled epizootological units are concentrated closer to Karaganda City, the reason for this is that there is the largest number of settlements, and, accordingly, animals, in this part of the raion. Similar trends can be seen in sampling performed in the Osakarovsky and Nurinsky Raions. Besides, the focus was made on the establishments where reports on IBR suspect cases had been previously submitted.

In order to determine seroprevalence against IBR virus, 285 sera samples were collected from non-vaccinated cattle in the Karaganda Oblast. A total of 21 epizootological units were studied in each of the 3 raions of the region. The results of the serological examination of the collected samples are shown in Figure 2.

The mean seroprevalence for non-vaccinated animals in 3 raions of the Karaganda Oblast was 79.29%. It should be noted that in the Abaisky and Osakarovsky Raions, where no IBR suspect cases were detected in 2021, the seroprevalence was 60.00 and 78.09%, respectively.

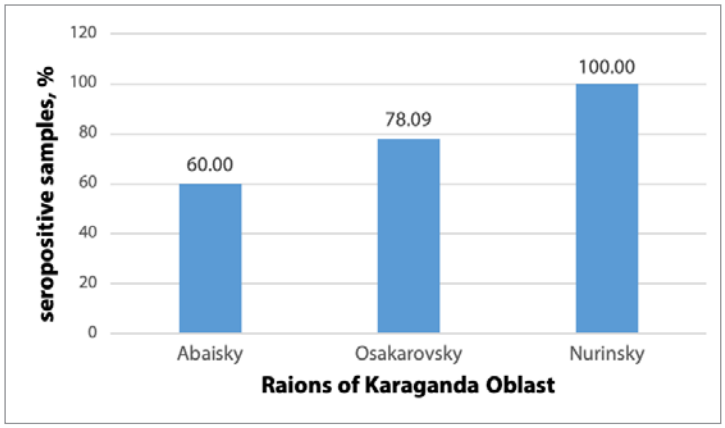

Fig. 2. Proportion of positive samples in the Karaganda Oblast by raions (2021)

Based on the plan of veterinary and preventive measures and due to the financing from the republican budget, the vaccination was carried out only in the localities that had previously submitted reports on IBR suspect cases in 2020 or 2021. The total proportion of vaccinated animals did not exceed 10–15% of the total cattle population in the raion. Cattle vaccinated with an immunoglobulin E vaccine were diagnostically tested, which made it possible to differentiate infected animals from vaccinated ones using the DIVA strategy.

Thus, the data obtained indicate a possible circulation of the IBR pathogen in some raions of the Karaganda Oblast in 2021.

In 2022, the scheduled monitoring was conducted in Bukhar-Zhyrausky, Aktogaisky and Karkaralinsky Raions of the oblast. In total, 21 epizootological units in each of the above-mentioned raions were studied and 285 bovine sera samples were tested for the presence of antibodies to the IBR virus.

The sera samples were obtained from clinically healthy non-vaccinated animals demonstrating no IBR clinical signs. The sample under study included the epizootological units where reports on IBR suspect cases had been previously submitted, as well as nearby settlements or driving areas. It should be noted that in order to achieve the maximum level of test reliability, settlements or driving areas with relative disease-freedom (IBR was never detected) were also examined for this infection.

As a result, IBR-positive animals were found in all epizootological units. The obtained data are visualized in Figure 3.

It was established that the minimum level of seroprevalence (66.66%) was noted in the Aktogaisky Raion, where animals were selected for testing from both backyards and large-scale farms of various forms of ownership.

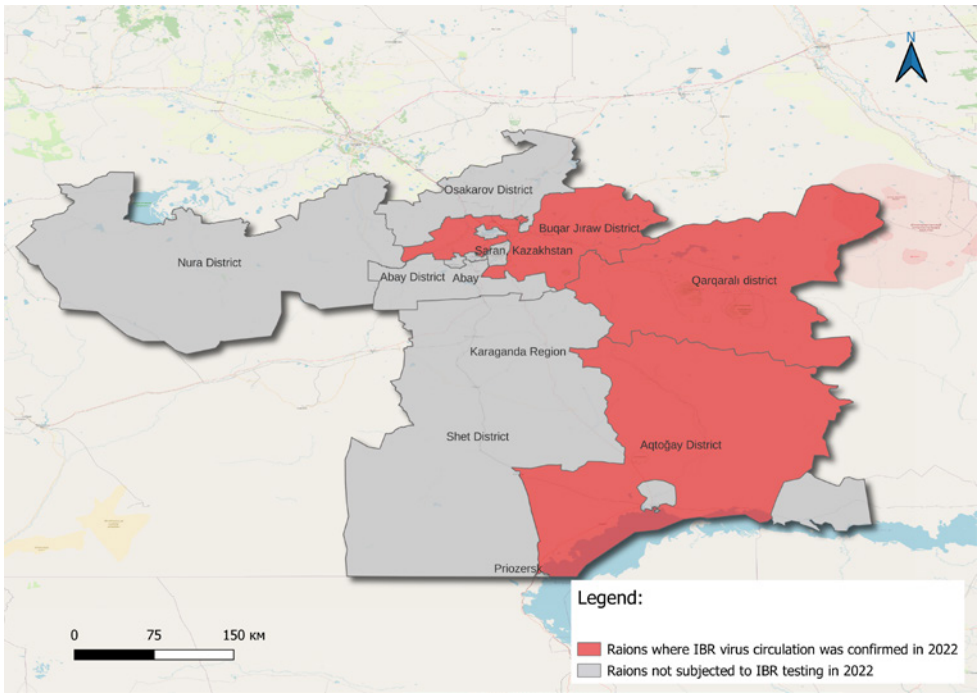

Fig. 3. Seroprevalence against IBR virus in 2022 (Karaganda Oblast)

The maximum level of 90% was recorded in the Karkaralinsky Raion. 97% of the animals tested in this area belonged to the owners who kept animals in backyards. Seroprevalence in the Bukhar-Zhyrausky Raion was 68.57%.

Considering that vaccination against IBR is carried out mainly in large-scale livestock farms and establishments, and immunization in the private sector is mainly financed from the republican budget, it can be confidently said that the chances of taking samples for testing from illegally vaccinated livestock are minimal.

In view of the above, it can be concluded that the circulation of the IBR virus was noted in all parts of the Karaganda Oblast under study in 2022.

CONCLUSION

The study of IBR epizootic situation in the Karaganda Oblast of the Republic of Kazakhstan demonstrated that the region was IBR-infected in 2021–2022. The first report on IBR possible occurrence in the oblast was received in 2013. The further spread of infection in the region was not immediate. Thus, not a single IBR suspicion case was recorded in the region in 2014. However, reports were made on possible occurrence of this infectious disease in animals in the region in 2015. The data analysis showed that since 2013 no cattle were imported from other epizootological units to the establishment where animals with IBR clinical signs were recorded. This fact may indicate a latent infection among livestock in the oblast. According to the data available, 7 IBR suspect cases were registered in the Karaganda Oblast in 2021. Testing of sera from clinically healthy and unvaccinated animals demonstrated high seroprevalence to the IBR agent in the Nurinsky, Abaiskysky and Osakarovsky Raions, where the proportion of vaccinated animals did not exceed 10–15% of the total livestock. In 2022 planned monitoring was carried out in Bukhar-Zhyrausky, Aktogaisky and Karkaralinsky Raions in the oblast, a total of 21 EUs were subjected to testing in each of the above-specified areas. The sample under study included the epizootological units with previously submitted reports on IBR suspect cases, as well as nearby settlements or driving areas. As a result, seropositive animals to the IRT causative agent were found in all EUs. Thus, the data obtained confirm the circulation of the IBR virus in the following raions of the Karaganda Oblast: Abaisky, Aktogaisky, Bukhar-Zhyrausky, Karkaralinsky, Nurinsky and Osakarovsky.

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Specific features of African swine fever control activities in China

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SUMMARY

African swine fever is a highly contagious viral disease of pigs; however, no vaccines are available to control it. Currently the disease causes significant economic damage in many countries. The Republic of China is the first country in Southeast Asia, which officially reported the African swine fever outbreak in 2018. The disease further spread to all provinces of the country with 200 outbreaks in domestic pigs and 10 outbreaks in wild boar in total; herewith the overall population of pigs decreased dramatically, approximately by 180 million animals. Following the confirmation of the first African swine fever outbreak, the PRC Ministry of Agriculture and Rural Affairs launched the "African Swine Fever Contingency Plan and Emergency Response", which facilitated the disease eradication and already by 2023, no new infection outbreaks had been reported. The country with the largest swine population in the world achieved rather moderate spread rates if compared to Europe. In addition, the virus strains, which circulated in the Chinese territory, were genetically diverse and different in virulence, leading to a wide range of clinical signs manifested by diseased animals. Such aspects were supposed to complicate the eradication measures, but instead, they proved their effectiveness. The experience gained from the disease control in China is most certainly of interest for the Russian Federation, infected with African swine fever since 2008.

Keywords: African swine fever, infection spread, epidemic situation, disease control measures, Republic of China

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Особенности реализации противоэпизоотических мероприятий по африканской чуме свиней в Китае

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РЕЗЮМЕ

Африканская чума свиней – вирусная болезнь свиней, обладающая высокой скоростью распространения, против которой не разработано средств специфической профилактики. Китайская Народная Республика является первой страной в Юго-Восточной Азии, где в 2018 г. была официально зарегистрирована вспышка данного заболевания, наносящего в настоящее время значительный экономический ущерб многим странам мира. Болезнь в дальнейшем распространилась на все провинции страны, где общее количество очагов среди домашних свиней составило 200, а в популяции диких кабанов – 10, при этом общее поголовье свиней в стране катастрофически сократилось – примерно на 180 млн голов. С момента выявления первого очага африканской чумы свиней Министерством сельского хозяйства и сельских дел Китая был принят «План действий в чрезвычайных ситуациях по борьбе с африканской чумой свиней и уровень реагирования на чрезвычайные ситуации», выполнение которого обеспечило ликвидацию эпизоотии, и уже к 2023 г. выявление новых очагов инфекции в стране прекратилось. Страна с одной из самых объемных свиноводческих отраслей животноводства в мире добилась, в сравнении с Европой, показателей довольно среднего распространения эпизоотии. При этом на территории Китая циркулировали штаммы вируса африканской чумы свиней с высоким генетическим разнообразием и с различным уровнем вирулентности, что обусловливало широкий спектр клинических симптомов у заболевших животных. Подобные особенности должны были только усложнить проведение ликвидационных мероприятий, однако разработанные меры доказали свою эффективность. Опыт осуществления противоэпизоотических мероприятий в Китае, безусловно, представляет интерес и для нашей страны, неблагополучной по африканской чуме свиней с 2008 г.

Ключевые слова: африканская чума свиней, распространение инфекции, эпизоотическая ситуация, противоэпизоотические мероприятия, КНР

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INTRODUCTION

Due to the recent global political and socio-economic situation in the Russian Federation, there is a clear trend towards a significant increase in contacts with Southeast Asia countries. In the veterinary field, from our perspective, these are primarily contacts concerning prevention and control of highly dangerous animal diseases, potentially causing significant economic losses in the livestock industry. In this respect, China's experience in control of African swine fever (ASF) is most certainly of interest because this devastating pandemic causes enormous damage to pig production in many countries of the world with no effective ASF vaccines been developed yet.

Interest in the peculiarities of the ASF disease control measures in China is primarily explained by the fact that it is a country with one of the largest pig production industries in the world, but at the same time the nation managed to minimize the spread of the disease in a relatively short period of time, whereas most European countries with modern highly developed livestock management systems have not yet coped with ASF spread in their territories.

Moreover, up to the present time, the infection is gradually moving to the west of Europe. ASF was reported in Poland, Lithuania, Estonia in 2014; in Moldova in 2016; in the Czech Republic and Romania in 2017; in Hungary, Bulgaria and Belgium in 2018; in Serbia and Slovakia in 2019. Later ASF outbreaks were reported by Greece and Germany in 2020, by Northern Macedonia in 2021, by Italy in 2022 [1].

According to Bloomberg News ASF outbreaks have spread throughout the European Union since 2014, at a speed of about 200 km per year, which, according to estimates, causes annual losses of several billion euros [2].

The purpose of this work is to review the Chinese experience in ASF outbreak eradication to highlight the key components of disease control measures for the Russian regional veterinary services.

MATERIALS AND METHODS

Foreign scientific literature was reviewed and publications from Chinese into Russian concerning ASF spread in China were translated.

Data on the epidemic situation were taken from the World Animal Health Organization (WOAH) official reports [1], including the date and place of ASF outbreaks, and the coordinates of infected settlements/areas for mapping.

Information on pig population density in China was provided by the PRC Ministry of Agriculture and Rural Affairs and was used to calculate the relative risk of new ASF outbreak occurrence in China.

Using ArcGIS software (Esri, USA), maps were created demonstrating ASF-infected countries, the infected Chinese provinces and the geographical locations of outbreaks. The risk of the disease spread in Southeast Asia was analyzed.

RESULTS AND DISCUSSION

The first ASF case in China was reported on August 1, 2018 [3], long after the disease had escaped from its endemic area in 1957–1970 and had widely spread in Europe and other regions (Fig. 1).

ASF outbreak in China is considered to be the first occurrence of the disease in Southeast Asia countries, as the subsequent outbreaks were reported later in Mongolia in January 2019, in Cambodia in March 2019, in Hong Kong and the DPRK in May 2019, in Laos in June 2019, in the Philippines in July 2019, in Myanmar in August 2019, in South Korea in September 2019, in Indonesia in November 2019, in Papua New Guinea in March 2020, in India in May 2020. ASF was reported in Bhutan, Malaysia, Thailand in 2021, and in Nepal in 2022.

Southeast Asia countries have the largest number of pigs in the world (Fig. 2); only China accounts for about 50% of the world pig population. At the same time, most of the farms in the country (80–90%) are small farms and backyards that produce not more than 500 pigs per year and are at extreme risk of infection introduction due to the lack of biosecurity measures. In 2017, 688.61 million pigs were raised in China, which is approximately 48% of global pork production, and already in 2019, there was a drop in production volumes down to 310.4 million pigs due to ASF occurrence [4].

Since the first official ASF report in August 2018 in China's northeastern Liaoning province, the disease has been rapidly spreading across the country [5]. 33 ASF outbreaks were reported in 7 provinces out of 22 provinces, 5 autonomous regions and 4 municipalities of mainland China as of October 8, 2018 [5, 6]: Anhui, Heilongjiang, Henan, Jilin, Liaoning, Jiangxi, Zhejiang and in the Inner Mongolia Autonomous Region (Fig. 3), but as of December 7, 2018, the disease also spread to the provinces of Guangdong, Fujian, Hubei, Shanxi, Yunnan and Sichuan, where 50 facilities (farms/slaughterhouses) were infected.

At the end of 2018, 102 ASF outbreaks were reported among domestic pigs and 2 among wild boars in 23 provinces / autonomous regions / municipalities of the country (Fig. 4).

The spread of infection continued in 2019. The provinces of Hunan, Guizhou, Jiangxi, Qinghai, Gansu and Shandong, as well as the Ningxia-Hui and Guangxi Zhuang Autonomous Regions and the municipalities of Tianjin,

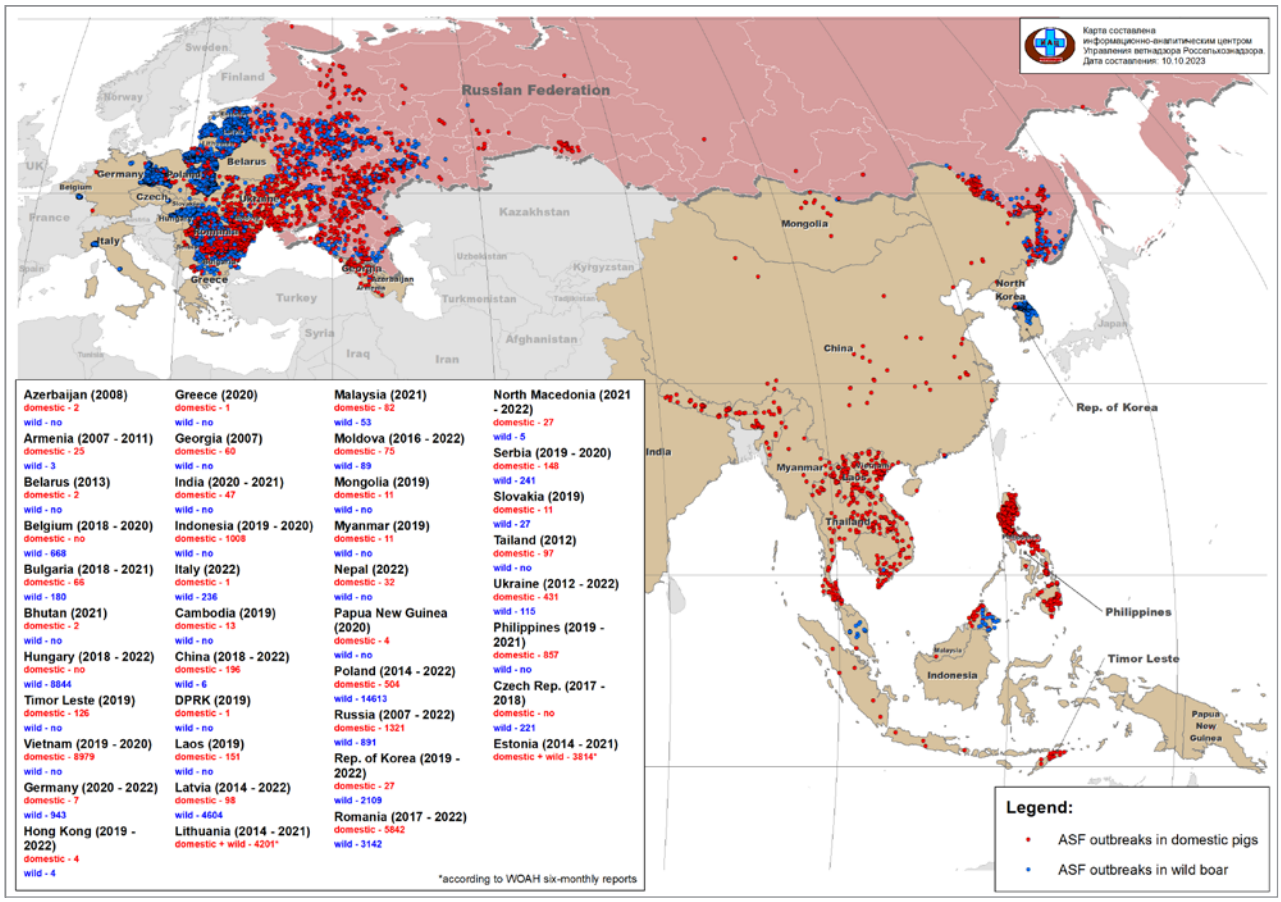

Fig. 1. ASF situation in the Russian Federation, European and Asian countries in 2007–2022 (based on urgent notifications to the WOAH as of November 30, 2022)

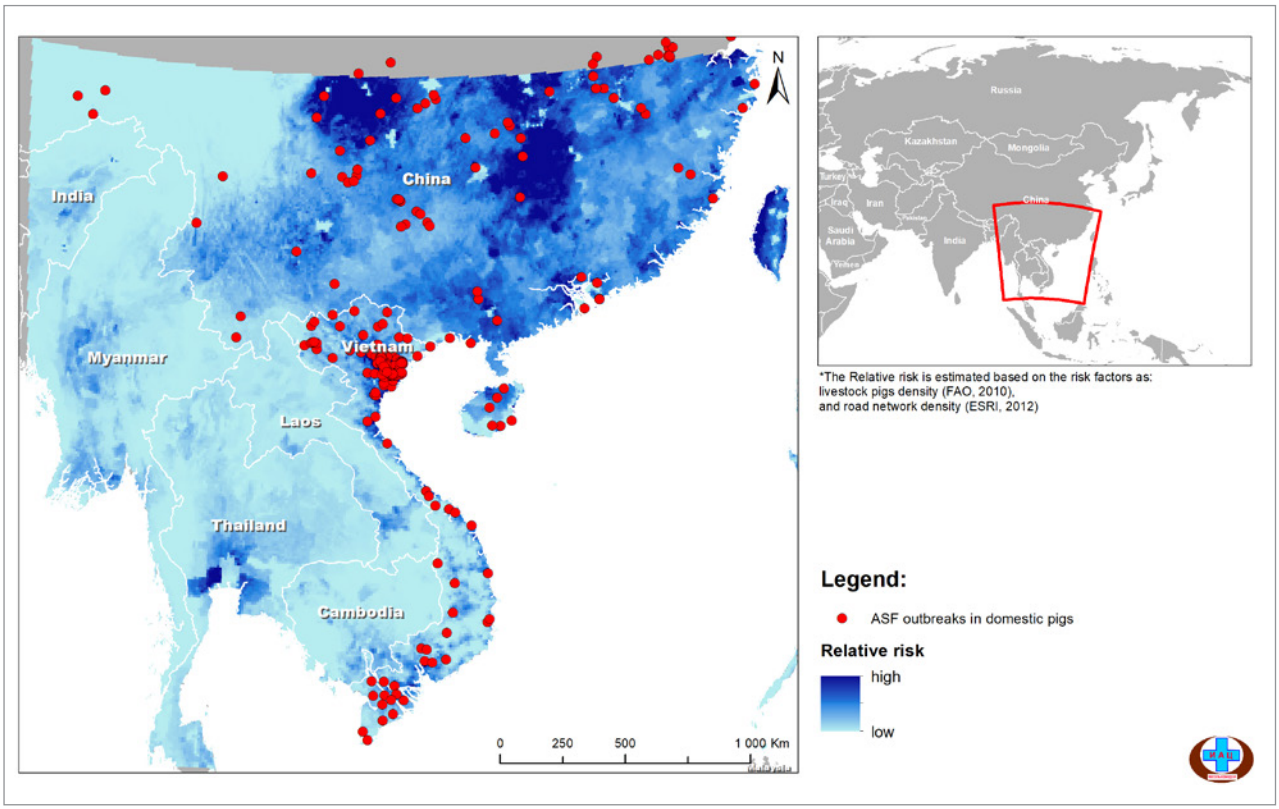

Fig. 2. Pig population density and relative risk of ASF spread in Southeast Asia countries

Chongqing, Shanghai and Beijing became also infected by ASF. In total, as of December 27, 2019, since the beginning of the ASF epidemic, the number of outbreaks reported in the country increased to 165; 4 of them among wild boars [7, 8].

In 2020, 33 outbreaks among domestic pigs and 2 among wild boars were detected in the same provinces as before, 4 cases of ASF among domestic pigs and 4 among wild boars were reported in 2021, and one ASF outbreak among domestic pigs in the Xinjiang Uygur Autonomous Region in 2022.

Thus, since the first occurrence of ASF in August 2018 and until 2023, the outbreaks were reported in almost all administrative regions of the country; the total number of outbreaks among domestic pigs was 200, and 10 outbreaks in the wild boar population (Fig. 5).

Compared with other, in particular European countries, the disease spread rate was fairly moderate, but, due to deaths of diseased animals in the outbreak areas and the policy of disease control measures adopted in China to prevent the infection spread by killing of pigs in the regions at risk, the total number of pigs in the country has catastrophically decreased by about 180 million animals.

One of the features of the epidemic in China was that highly genetically diverse ASFV strains with different levels of virulence circulated in the country, thus causing a wide range of clinical symptoms in diseased animals.

Studies by X. Wen et al. [9] demonstrated that the virus genome responsible for the outbreak in China in 2018 (China 2018/1) was mostly similar to the genome of the ASF virus isolated in Poland (GenBank: MG939588.1).

Based on the results of phylogenetic analysis of 66 strains isolated in 2019–2020 in the southern province of Guangxi, they were grouped into 8 different

Fig. 3. ASF infected regions of China in August – October 2018 [5]

variants, with 3 of them belonging to genotype 1; and 6 belonging to genotype 2 (p72), serogroup 8 (CD2v) [10]. Two non-haemadsorbing ASFV strains (HeN/ZZ-P1/21 and SD/DY-I/21), belonging to genotype 1 (with low virulence and causing chronic disease in pigs) were isolated in the provinces of Henan and Shandong [11–13].

The Chinese strains with reduced pathogenicity belonging to genotype 1 were identical to the strains isolated in the 60s of the last century in Portugal and Spain, and genotype 2 virus circulating in China since 2018 was similar to highly virulent ASFV isolates Georgia 2007/1, Krasnodar 2012, Estonia 2014 [10, 14, 15].

Fig. 4. ASF situation in China in 2018

Fig. 5. ASF situation in China from August 2018 to January 23, 2023

Since the identification of the first ASF outbreak, the PRC Ministry of Agriculture and Rural Affairs launched the African Swine Fever Contingency Plan and Emergency Response [16], providing for a set of the disease control measures. The Government established a 3-km protection zone and 10-km surveillance zone around the outbreak areas. Strict rules and measures, including quarantine, restrictions of pig and pig products movements within the country, surveillance outside the animal keeping facilities, mandatory culling of all diseased and contact pigs within a radius of 3 km from the infected area followed by carcass disposal.

An intersectoral interaction mechanism was created, which coordinated various agencies in ASF control and prevention; a multidisciplinary program of epidemiological monitoring was elaborated, covering the development of diagnostic tests for specific and early detection of animal cases, genetic analysis of isolated viral isolates, as well as monitoring among wild boar and tick collection. Compensation for case notifications, animal deaths and emergency slaughter of up to 1,200 yuan per animal (about 175 US dollars) was introduced everywhere.

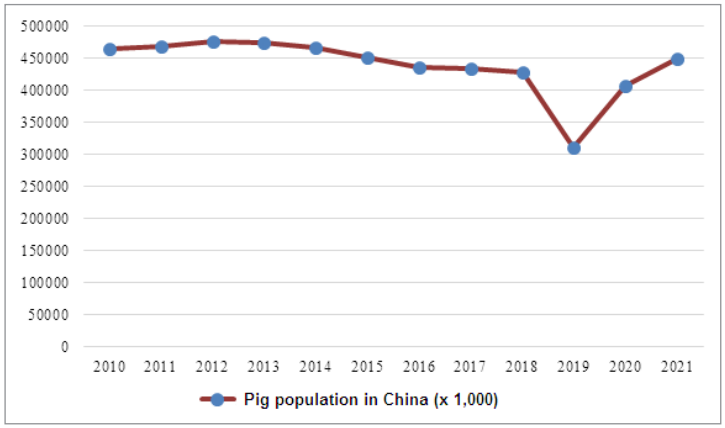

Fig. 6. Pig population numbers in China in 2010–2021 [19]

The measures taken and, first of all, in our opinion, various restrictions, pig culling in infected and protection zones, a flexible system of compensation payments, etc., facilitated a significant improvement in the ASF situation in China, and already in February 2020, the Chinese authorities announced that the ASF situation in the country stabilized [16], and by 2023, no new ASF outbreaks in the country were reported, although, according to Krasnaya Vesna News Agency a single ASF outbreak was registered in Hong Kong and several outbreaks in the provinces of Shandong and Hebei in February – March 2023, where the disease in animals was clinically mild [17, 18].

Moreover, by the end of 2021 (Fig. 6) the number of pig population in the country almost recovered, amounting to 449.2 million animals [19].

CONCLUSION

Experience of ASF eradication in China shows that the fight against this devastating pandemic can be successful throughout the country even without the vaccine use, but it must include strict sanitary restrictions associated with huge financial expenditures. At the same time, each country has developed its own control methods that can be applied in other countries too. We believe that the Chinese experience in ASF control measure implementation, taking into account the circulation of the ASFV 1 and 2 genotypes on its territory, should be thoroughly analyzed in Russia to be potentially used in such regions as,

for example, the Far East. First of all, in this regard, China's experience in implementation of a strict centralized strategy for ASF control and prevention throughout the country, significant enhancing of local authorities' role in arrangement and supervision of general and specific disease control measures, introduction of a well-arranged compensation system in case of pig culling to prevent the infection spread is of great interest.

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Joint use of polyvalent serum and immunomodulators for calves in early postnatal period

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SUMMARY
The paper demonstrates how a joint use of polyvalent serum and immunomodulators affects morbidity, survival, and resistance of calves in the early postnatal period. The objectives of the research are: to study how a joint administration of polyvalent serum and immunomodulators changes dynamics of morpho-biochemical and immunological blood parameters in calves; to determine an optimal ratio between the polyvalent serum and immunomodulators and frequency of administration so that to increase overall body resistance, ensure survival of calves in the early postnatal period; to assess cost-effectiveness of the joint use of polyvalent serum and immunomodulators. Research and production testing was done in Simmental calves. For this purpose one control group and five experimental groups were formed (at least 5 animals in each group). Polyvalent serum (20.0 mL) was once administered subcutaneously to the control calves on the first day of life and the animals of the experimental groups received the serum and immunomodulators according to the relevant dosing instructions. The obtained results demonstrate that the optimal protocols include a single administration of "Ribotan" in combination with serum on the first day of life, as well as a double administration of a polyvalent serum 7 days later, alternating "Fosprenil" with "Immunophane". Administration of polyvalent serum together with an immunomodulator to newborn calves can reduce the morbidity by 70.0% and achieve 100.0% survival, improving this indicator by 22.3% compared to the control. Cost-effectiveness assessment of the veterinary measures specified in these protocols shows that each rouble spent on the measures saves 25.29 roubles. To increase nonspecific resistance of calves in the early postnatal period, preference shall be given to those veterinary medicinal product that can not only normalize the immune system, but also have a combined positive effect on homeostasis in general, stimulate the growth and development of young animals at the early stages of ontogenesis.

Keywords: immunomodulators, polyvalent serum, calves, morbidity, survival, resistance

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Сочетанное применение поливалентной сыворотки и иммуномодуляторов в ранний постнатальный период выращивания телят

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РЕЗЮМЕ
Представлены данные о влиянии сочетанного применения поливалентной сыворотки и иммуномодулирующих препаратов на заболеваемость, сохранность, резистентность в ранний постнатальный период выращивания телят. Задачами исследования было: изучение влияния комбинированного введения поливалентной сыворотки и иммуномодуляторов на динамику морфобиохимических, иммунологических показателей крови телят; определение оптимального сочетания, кратности введения поливалентной сыворотки и иммуномодуляторов для повышения общей резистентности организма, сохранности телят в ранний постнатальный период выращивания; расчет экономической эффективности применения поливалентной сыворотки и иммуномодуляторов. Научно-производственный опыт проведен на телятах симментальской породы. Для этого сформировали одну контрольную и пять опытных групп, содержащих не менее 5 особей в каждой. Телятам контрольной группы однократно подкожно в первый день жизни вводили поливалентную сыворотку в дозе 20,0 мл, животным опытных групп – сыворотку и иммуномодуляторы в дозах согласно наставлению по их применению. На основании полученных результатов сделан вывод, что оптимальными протоколами являются: однократное введение в первый день жизни «Риботана» в комбинации с сывороткой, а также двукратное введение через 7 дней поливалентной сыворотки с чередованием «Фоспренила» и «Иммунофана». Инъекции новорожденным телятам поливалентной сыворотки в комбинации с иммуномодулятором позволяют снизить заболеваемость телят на 70,0%, добиться 100,0%-й сохранности, улучшив данный показатель на 22,3% по сравнению с контролем. Экономическая эффективность ветеринарных меро-

приятий в результате использования указанных протоколов на 1 рубль затрат в среднем составила 25,29 рубля. Для повышения уровня неспецифической резистентности организма телят в ранний постнатальный период выращивания предпочтение необходимо отдавать препаратам, способным не только нормализовать работу иммунной системы, но и оказывать комплексное положительное воздействие на гомеостаз в целом, стимулировать рост и развитие молодняка на ранних этапах онтогенеза.

Ключевые слова: иммуномодуляторы, поливалентная сыворотка, телята, заболеваемость, сохранность, резистентность

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INTRODUCTION

Modern animal husbandry inevitably causes problems with feeding and keeping newborn calves. These problems result in gastrointestinal disorders and bronchopneumonia, followed by reduced nonspecific resistance and immunodeficiencies in animals [1–3]. High morbidity and mortality rates during the first days of life are primarily explained by the undeveloped self-regulatory mechanisms of the body, imperfect digestion and poor immunity [4, 5]. The solution here is to develop comprehensive and effective protocols that both optimize the work of the immune system and have a positive effect on the body as a whole, activate metabolic processes, homeostasis, growth and development of newborn calves [6, 7]. Modern medicine and veterinary science offer quite a lot of natural and synthetic pharmacological products to stimulate immunogenesis and general resistance of the animals. However, their limited use for veterinary purposes indicates that no effective or simple ways of pharmacological influence on the animal immune status have been found so far [8]. Ensuring a high level of protection and adaption of animals to negative environmental factors during critical early postnatal periods remains an urgent issue. Creation of new and improvement of the current schemes for the use of polyvalent serum together with immunomodulators will optimize the protocols for the production purposes.

The aim of the research was to study the effect of the joint use of polyvalent serum with an immunomodulator on morbidity, survival, and resistance of calves during the early postnatal period.

To achieve this goal, the following tasks were set:

1. To study the effect of joint administration of polyvalent serum and immunomodulators on dynamics of morpho-biochemical, immunological blood parameters.

2. To determine optimal ratio and frequency of administration of the polyvalent serum and immunomodulators to increase general resistance and survival rate of calves in the early postnatal period.

3. To analyze cost-effectiveness of the joint use of polyvalent serum and immunomodulators in the early postnatal period.

MATERIALS AND METHODS

Research and production testing was conducted on one of the farms of the Altai Krai to study the joint use of a polyvalent serum (against pasteurellosis, salmonellosis, escherichiosis, parainfluenza-3 and infectious bovine rhinotracheitis; Armavir Biofactory, Russia) and immunomodulators: "Fosprenil" (CJSC "Micro-Plus", Russia), "Mixoferon" (AO "Mosagrogen", Russia), "Immunophane" (OOO NPP "BIONOX", Russia), "Ribotan" (OOO firm "NPViZTS "VETZVEROTSENTR", Russia). For this purpose, animal groups were formed: a control group (K-1) and 5 experimental (O-1–O-5) groups of Simmental calves (at least 5 animals in each). Polyvalent serum (at a dose of 20.0 mL) was once administered intramuscularly to the control animals, on the first day of life. The calves of the experimental groups were given serum and immunomodulators according to the scheme given in Table 1. The animals were kept under the same conditions and had the same diet.

Effectiveness of the joint use of immunomodulators and polyvalent serum was assessed based on the results of the following tests: tests for erythrocyte and leukocyte total count, test for hemoglobin (using conventional methods [9]); sera biochemical test: total protein (refractometric analysis, IRF-22), protein fractions (nephelometry) [10]; bactericidal (BAS) and lysozyme (LAS) activity of sera (photonephelometric method) [11]; methemoglobin concentration in blood (by colorimetric method) [12]; total IgG levels (enzyme immunoassay using the appropriate kit); phagocytic activity of neutrophils (by the methods of A. I. Ivanov and B. A. Chukhlovin [13] using a test culture of *Escherichia coli* O111 grown during one day on meat-peptone agar; test for T- and B-lymphocyte levels in peripheral blood using spontaneous rosettes with sheep erythrocytes and mouse erythrocytes according to S. V. Burtseva and O. Yu. Rudishin [14]; cost-effectiveness was calculated according to method offered by Yu. E. Shatokhin et al. [15].

Blood was sampled before administration of serum and immunomodulators and 10 days after the administration.

All experiments were carried out in strict accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS No. 123).

Table 1
Scheme of joint administration of polyvalent serum against associated bovine infections and immunomodulators to calves

Group	Number of animals	Frequency / interval between injections, days	Veterinary medicinal product, mL				
			polyvalent serum	"Ribotan"	"Mixoferon", doses	"Fosprenil"	"Immunophane"
K-1	9	1	20.0	–	–	–	–
O-1	5	1	20.0	1.0	–	–	–
O-2	5	2/14	20.0	–	–	2.5	–
O-3	5	2/14	20.0	–	5	–	–
O-4	5	2/7	20.0	–	–	2.5	1.0
O-5	5	2/7	20.0	–	–	–	1.0

The reliability of the mean values was evaluated by method of variational statistics according to the Student – Fisher criterion.

RESULTS AND DISCUSSION

Analysis of the basic blood parameters in newborn calves before administration of serum and immunomodulators on the first day of life revealed that the total amount of serum protein was lower than the reference limit [16] by 17.3%, alpha-globulin fraction – by 19.1%, IgG was absent in 40.0% of experimental animals (Table 2).

Blood tests conducted at the end of the experiment demonstrated a significant decrease in total protein in the control group by 28.0% ($P < 0.05$) in contrast to the baseline parameters. A significant increase in this parameter in comparison with this parameter in the controls was recorded in groups O-1–O-5 with a simultaneous decrease in the serum albumin within the normal range in all experimental groups, except for O-3. Serum alpha-globulin fraction in all experimental calves demonstrated a positive trend towards normalization with a significant increase in O-2 group ($P < 0.05$). A decrease in serum gamma-globulin fraction in comparison with the controls was detected in groups O-1–O-3 ($P < 0.05$), groups O-4 and O-5 demonstrated a 22.5% and 11.8% increase in this parameter ($P < 0.01$), respectively. An increase in total IgG by a factor of 2.2 and by 32.5% in comparison to the baseline parameters was noted in sera of calves from groups O-3 and O-4, respectively, at the same time IgG was detected in blood of 100.0% of the examined animals. A maximum IgG increase in comparison to the controls was recorded in group O-3 (by a factor of 3.4), in group O-4 (by a factor of 2.0), in group O-5 (by 34.6%), an IgG decrease was observed in groups O-1 and O-2 by 38.4% and 19.2%, accordingly. The albumin-globulin ratio was within the normal physiological range only in groups O-2 and O-3.

The hematological profile of the calves used in the experiment is given in Table 3. Analyses of the baseline blood parameters revealed that the total count of erythrocytes and leukocytes was within the reference range, while the total hemoglobin was 21.8% lower than normal, the color index was 2.8%. Experimental calves

Table 2
Total protein and its fractions in the sera of experimental calves

Group	Total protein content, g/L	Albumins, %	Globulins, %			Total IgG, Units/mL	Ratio albumin/ globulin, Units
			α	*β*	*γ*		
Reference	60–85	33–50	12–20	10–16	25–40	< 10	0.83–1.19
baseline blood parameters							
	49.6 ± 3.93	42.6 ± 6.65	9.7 ± 2.10	16.1 ± 3.89	30.7 ± 7.90	4.0 ± 2.51	0.72 ± 0.163
in 10 days							
K-1	35.7 ± 1.49*	46.9 ± 5.74	11.2 ± 1.57	16.0 ± 3.96	25.3 ± 2.32	2.6 ± 0.94	0.73 ± 0.090
O-1	64.2 ± 4.72**(*)	35.7 ± 1.50	11.7 ± 1.86	17.0 ± 0.94	17.0 ± 0.94(*)	1.6 ± 0.52	0.55 ± 0.040
O-2	44.5 ± 0.98(*)	44.4 ± 0.94	16.7 ± 0.39*	12.9 ± 1.33	17.0 ± 0.94(*)	2.1 ± 1.01	0.83 ± 0.058
O-3	47.0 ± 1.70(*)	47.0 ± 1.82	13.8 ± 1.17	13.4 ± 1.59	15.1 ± 0.41(*)	8.8 ± 1.77(**)	0.89 ± 0.060
O-4	57.8 ± 1.49(*)	35.6 ± 4.51	11.5 ± 2.36	14.5 ± 1.52	31.0 ± 0.42(**)	5.3 ± 2.14	0.51 ± 0.030
O-5	49.7 ± 0.41(*)	41.8 ± 3.53	12.9 ± 1.77	16.9 ± 2.13	28.3 ± 4.48	3.5 ± 2.32	0.73 ± 0.101

* $P < 0.05$, ** $P < 0.01$ – to the initial values;
(*) $P < 0.05$, (**) $P < 0.01$ – to control.

Table 3
Hematological parameters observed in experimental calves before and after administration of immunomodulators together with polyvalent serum

Group	Erythrocytes, 10^9/L	Hemoglobin, g/L	Methemoglobin, %	Leukocytes, 10^{12}/L	Color index, Units	BAS, %	LAS, %
Reference	5.0–7.5	99–129	0–5	4.5–12.0	0.7–1.1	23–28	25–33
baseline blood parameters							
–	6.7 ± 0.80	77.4 ± 6.13	20.0 ± 0.62	5.5 ± 1.21	0.68 ± 0.10	33.3 ± 9.86	5.2 ± 0.37
blood parameters at the end of the experiment in 14 days							
K-1	7.7 ± 0.64	79.0 ± 3.09	18.1 ± 0.91	5.4 ± 0.42	0.71 ± 0.06	34.3 ± 4.47	6.7 ± 1.45
O-1	9.3 ± 0.47(*)	90.5 ± 2.33(**)	0	5.4 ± 0.54	0.62 ± 0.06	41.8 ± 1.6**	21.2 ± 3.10*(*)
O-2	8.0 ± 0.04	82.5 ± 2.89	19.7 ± 0.22	7.5 ± 0.47(*)	0.61 ± 0.01	34.3 ± 4.29	15.5 ± 1.36*
O-3	7.7 ± 0.44	107.3 ± 6.42(*)	13.5 ± 0.6*(*)	6.5 ± 0.65	0.82 ± 0.03	46.3 ± 6.95	20.8 ± 0.88*(*)
O-4	8.4 ± 0.75	85.5 ± 4.28	13.1 ± 0.75*(*)	5.8 ± 0.28	0.7 ± 0.09	45.7 ± 6.82	20.2 ± 0.95*(*)
O-5	8.1 ± 0.50	88.7 ± 3.75	17.7 ± 0.28*	6.8 ± 1.01	0.65 ± 0.06	38.2 ± 4.50	14.9 ± 0.26*(*)

* P < 0.05, ** P < 0.01 – to the initial values;
(*) P < 0.05, (**) P < 0.01 – to control.

demonstrated a 4-time increase in methemoglobin in comparison with the reference limits, BAS – by 18.9%, and a decrease in LAS – by 79.2%.

At the end of the experiment, a significant increase in total erythrocyte count was reported in group O-1 by 20.8% (P < 0.05), group O-2 by 3.9%, group O–4 by 9.1%, group O-5 by 5.2%, as compared to the control. Total hemoglobin significantly increased as compared to the baseline parameters and to the blood tests in the control group: i.e. in O-1 group by 14.6% (P < 0.01) and in O-3 group by 35.8% (P < 0.05). A decrease in total methemoglobin was observed in groups O-3–O-5 (P < 0.05) with a significant difference, thus, suggesting a decreased tissue hypoxia due to reduced levels of oxidized trivalent iron in blood of experimental animals. The leukocytes were within the reference limits. In comparison with the control group, the color index grew by 15.5% in group O-3 and decreased by 1.4–14.1% in groups O-1, O-2, O-4, O-5. BAS grew in group O-1 by 21.9% (P < 0.01), in groups O-3 – by 35.0%, O-4 – by 33.2%, O-5 – by 11.4% relative to the control. LAS in all experimental groups significantly increased by a factor of 2.2–3.2 (P ≤ 0.05) in comparison with the parameter observed in the control group.

Functional immune system in the experimental calves was assessed and the corresponding results are given in Table 4. Increased proliferation activity of T-lymphocytes with a significant difference (P ≤ 0.05; P ≤ 0.01) in relation to the baseline parameters was reported in all animals: in K-1 group this parameter increased by 17.7%, in the experimental groups – by 22.8–29.9%. The B-lymphocytes count significantly differed only in groups O-1 and O-4 (P ≤ 0.05). The phagocytic activity of neutrophils increased in the control group by 11.9%, in the experimental group – by 14.6–21.8% (P ≤ 0.05) in comparison with the baseline parameters. Increased number of phagocytic cells by 15.4–30.8% was found in the blood of experimental calves without any significant differences.

Table 4
Functional activity of blood cells in calves before and after the combined administration of immunomodulators and polyvalent serum

Group	B-lymphocytes, %	T-lymphocytes, %	Phagocytic activity, %	Phagocytic number, %
baseline parameters	11.2 ± 1.48	25.4 ± 0.35	55.4 ± 1.56	1.3 ± 0.13
blood parameters at the end of the experiment, in 14 days				
K-1	15.6 ± 2.43	29.9 ± 2.65*	62.0 ± 2.22	1.5 ± 0.16
O-1	20.4 ± 2.50*	33.0 ± 1.75*	67.5 ± 2.26*	1.6 ± 0.14
O-2	16.6 ± 2.13	32.8 ± 2.21*	65.1 ± 1.96*	1.5 ± 0.12
O-3	17.8 ± 2.48	31.2 ± 1.32*	64.2 ± 1.26*	1.6 ± 0.13
O-4	17.8 ± 1.72*	32.2 ± 1.28**	65.3 ± 2.48*	1.7 ± 0.15
O-5	16.4 ± 2.55	32.0 ± 2.21*	63.5 ± 2.35*	1.5 ± 0.21

* P ≤ 0.05, ** P ≤ 0.01.

The joint administration of serum with "Ribotan" (O-1), serum with "Fosprenil" and "Immunophane" (O-4) to newborn calves made it possible to achieve 100.0% survival in these groups, which is by 22.3% higher than in the control group (K-1), by 20.0% – in comparison with groups O-2, O-3 and O-5, where "Fosprenil", "Mixoferon" and "Immunophane" were injected together with the serum (Fig. 1).

The morbidity in the control group was 100.0%, in groups O-1 and O-4 – 20.0%, in O-2 and O-5 – 40.0%, and in group O-3 – 60.0%, average morbidity in experimental groups was 36.0%.

The 1st control weighing (see Fig. 2) shows that an absolute body weight gain in calves increased by 64.2% in group O-1; by 59.9% in group O-2; by 1.8% in group O-3 and by 75.6% in group O-5; a decrease in this indicator by 24.1% was reported in group O-4 as compared to the control (K-1). The trend continued during the whole 3-month observation with the maximum body weight gain by 19.1% in group O-5; by 1.6–15.1% – in O-1–O-3 groups and a decrease by 6.4% in O-4 group in comparison with the controls.

Cost-effectiveness of the joint use of immunomodulators and polyvalent serum against bacterial and viral infections to increase nonspecific resistance in calves in the early postnatal period was calculated as the difference between the probable (potential) and actual economic damage prevented on the farm. Calculations were based on the data obtained in the research and production test.

The damage caused by the death of calves was calculated by the formula:

$$Y_1 = M \times (Sp + Vp \times T \times C) - Sf,$$

where M – the number of dead animals; Sp is the cost of the offspring at birth (roubles), Sp = 3.61 × Cp, where 3.61 is the amount of milk (c) that can be produced from feeds consumed for the fetus formation, Cp is the purchase price of one centner of whole milk (roubles): Sp = 3.61 × 2,900 = 10,469.00 roubles; Vp – daily average body weight gain in young farm animals, kg; T is the age of the dead animal, day; C – the sale price for a unit of product, roubles; Sf – proceeds from the sale of slaughter products, roubles.

Y_1 (K-1) = 2 × (10,469.00 + 0.333 × 7 × 175) – 380 = 21,373.85 roubles;

Y_1 (O-2) = 1 × (10,469.00 + 0,531 × 3 × 175) – 180 = 10,567.78 roubles;

Y_1 (O-3) = 1 × (10,469.00 + 0.338 × 7 × 175) – 175 = 10,708.05 roubles;

Y_1 (O-5) = 1 × (10,469.00 + 0.583 × 4 × 175) – 165 = 10,712.10 roubles;

Y_1 (O-1, O-4) = 0 roubles.

Calculation of the damage caused by a decrease in the productivity of calves:

$$Y_2 = Ms \times (Vh - Vs) \times T \times C,$$

where Ms – number of sick animals; Vh and Vs – daily-average productivity of healthy and sick animals, kg; T – average observation of changes in animal productivity, days, C – sales price for a unit of product, roubles.

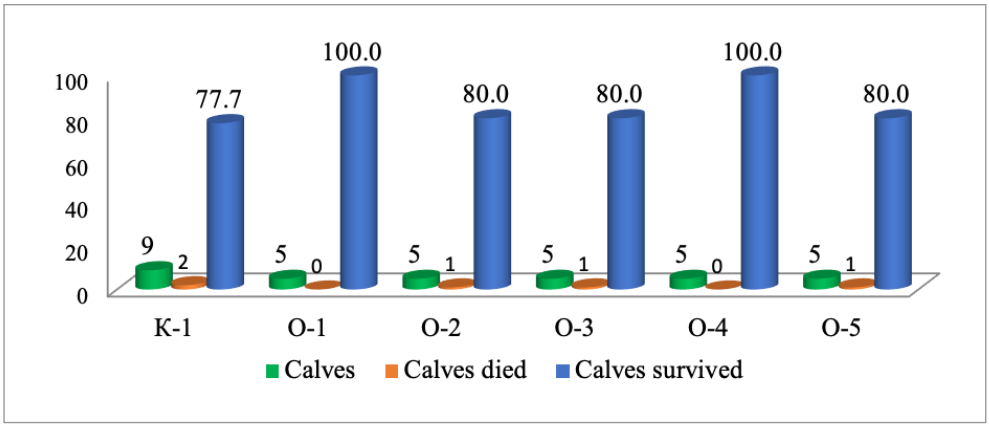

Fig. 1. Calves survived after joint administration of immunomodulators and polyvalent serum

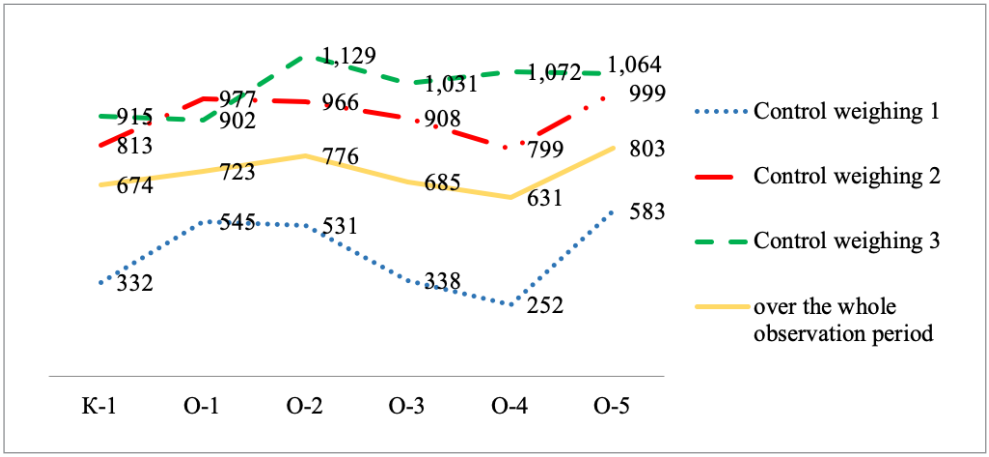

Fig. 2. Absolute weight gain in grams

Y_2 (K-1) = 9 × (0.332 – 0.213) × 4.5 × 175 = 843.41 roubles;

Y_2 (O-1) = 1 × (0.545 – 0.429) × 3.5 × 175 = 71.05 roubles;

Y_2 (O-2) = 2 × (0.531 – 0.492) × 4 × 175 = 54.60 roubles;

Y_2 (O-3) = 3 × (0.338 – 0.251) × 5 × 175 = 228.38 roubles;

Y_2 (O-4) = 1 × (0.252 – 0.215) × 4 × 175 = 25.90 roubles;

Y_2 (O-5) = 2 × (0.583 – 0.432) × 4 × 175 = 211.40 roubles.

Total damage and damage per 1 animal, roubles:

$Y = Y_1 + Y_2$.

Y (K-1) = 21,373.85 + 843.41 = 22,217.26 / 9 = 2,468.58 roubles;

Y (O-1) = 0 + 71.05 = 71.05 / 5 = 14.21 roubles;

Y (O-2) = 10,567.78 + 54.60 = 10,622.38 / 5 = 2,124.48 roubles;

Y (O-3) = 10,708.05 + 228.38 = 10,936.43 / 5 = 2,187.29 roubles;

Y (O-4) = 0 + 25.90 = 25.90 / 5 = 5.18 roubles;

Y (O-5) = 10,712.10 + 211.40 = 10,923.50 / 5 = 2,184.70 roubles.

The cost of one animal treatment, roubles: K-1 = 20.00 roubles, O-1 = 99.90 roubles, O-2 = 54.05 roubles, O-3 = 51.75 roubles, O-4 = 70.82 roubles, O-5 = 87.60 roubles.

Cost-effectiveness of one animal treatment:

$$Ec = (Sb + Ub) – (Sn + Un),$$

where Sb and Sn stand for the current production costs for veterinary measures, respectively, in the basic and new versions per treated animal, roubles; Ub and Un stand for a specific economic damage per unit of work, respectively, in the basic and new versions, roubles.

Ec (O-1) = (0 + 2,187.88) – (99.90 + 14.21) = 2,073.77 roubles;

Ec (O-2) = (0 + 2,187.88) – (54.05 + 2,124.48) = 9.35 roubles;

Ec (O-3) = (0 + 2,187.88) – (51.75 + 2,187.29) = –51.16 roubles;

Ec (O-4) = (0 + 2,187.88) – (70.82 + 5.18) = 2,111.88 roubles;

Ec (O-5) = (0 + 2,187.88) – (87.60 + 2,184.70) = –84.42 roubles.

The economic effect of preventive measures calculated per one rouble of expenses according to the formula:

$$Ee = Ec / Zv,$$

where Ec – cost-effectiveness of one animal treatment; Zv – veterinary costs per one animal, roubles.

O-1 = 2,073.77 / 99.90 = 20.76 roubles;

O-2 = 9.35 / 54.05 = 0.17 roubles;

O-3 = -51.16 / 51.75 = –0.99 roubles;

O-4 = 2,111.88 / 70.82 = 29.82 roubles;

O-5 = -84.42 / 87.60 = –0.96 roubles.

The economic effect of preventive measures calculated per one rouble of expenses in the experimental groups ranged from 0.17 roubles in group O-2, to 29.82 roubles in group O-4, negative values were obtained in groups O-3 and O-5.

The joint use of polyvalent serum and immunomodulators shows that the use of "Ribotan" together with serum (O-1), administered once on the first day of life, is the most effective option. The following scheme ranks second among other options, i.e. double administration of a polyvalent serum with "Fosprenil" and "Immunophane" (O–4) used on a rotational basis (with a 7-day interval). These two immunomodulators activate immunity of calves, which is confirmed by a significant increase in T- and B-lymphocytes and by growing phagocytic activity of neutrophils. It should be noted that the injection of a polyvalent serum into newborn calves in combination with immunostimulants reduces mortality of calves by 70.0%, helped to achieve 100.0% survival, improving this indicator by 22.3% in comparison to the control. Therefore, all other things being equal during the use of polyvalent serum together with immunomodulators, those veterinary medicinal products shall be preferred that can both normalize the immune system and have a complex positive effect on homeostasis in general, stimulate growth and development of young animals in the early stages of ontogenesis.

CONCLUSIONS

1. A single intramuscular injection of polyvalent serum at a dose of 20.0 mL to newborn calves together with a subcutaneous injection of "Ribotan" at a dose of 1.0 mL normalizes morpho-biochemical blood parameters with a significant increase in total hemoglobin, erythrocytes, serum protein, gamma globulin fraction, T- and B-lymphocytes, phagocytic activity of neutrophils, lysozyme and bactericidal activity of sera in the early postnatal period.

2. A double administration of the polyvalent serum at the same dose with "Fosprenil" (which is administered on Day 1 at a dose of 2.5 mL) and "Immunophane" (which is administered 7 days later at a dose of 1.0 mL) significantly reduces methemoglobin in the blood of calves, and increases total protein, gamma globulins, T- and B-lymphocytes, phagocytic lysozyme activity in comparison with the control calves and the baseline blood parameters.

3. These schemes make it possible to reduce morbidity of calves in early ontogenesis by 70.0%, to achieve a reduction in mortality by 22.3%.

4. Cost-effectiveness assessment of the veterinary measures specified in protocols 1 and 2 used to improve nonspecific resistance of calves in the early postnatal period shows that each rouble spent on the measures saves on average 25.29 roubles.

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Functional morphology of digestive organs of newborn calves and pathogenesis of escherichiosis

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SUMMARY

Despite numerous studies, the problem of escherichiosis in newborn calves remains one of the most urgent due to the extensive spread of the disease and high mortality of young animals. This paper presents results of experimental studies carried out at the FSBSI "All-Russian Veterinary Research Institute of Pathology, Pharmacology and Therapy". The aim of the work was to conduct complex morphological studies using modern methods for a deeper understanding of escherichiosis etiology and pathogenesis. For that, pathological samples were collected from 28 1–10 day-old calves diagnosed with colibacteriosis at the initial disease stage, demonstrating pronounced clinical signs and having the terminal stage of the disease. Samples from 6 clinically healthy calves of a similar age were used as control. It was found that newborn calves at the initial stage of escherichiosis demonstrated early structural changes in the ultrastructure of mucosa cells of the rennet and small intestine, as well as in the liver parenchyma, and mild changes – in the exocrine part of the pancreas. The most profound morphofunctional changes were observed in digestive organs with apparent clinical signs of the disease. As the condition developed, the range of pathological processes expanded and involved the structural organization of the rennet, small and large intestines, liver and pancreas. At the terminal stage of escherichiosis, deep inflammatory processes occurred not only in digestive organs, but also in other systems of diseased calves. Structural changes in digestive organs had an alterative nature at the initial disease stage, whereas in case of clinically pronounced disease signs there were manifestations of catarrhal-necrotic inflammation with multiple hemorrhages in the gastrointestinal tract and parenchymal organs. Digestive organ pathology plays the leading role in formation of the clinical and morphological picture at the initial stage of escherichiosis in calves. As the disease developed, the calves demonstrated changes at molecular and subcellular levels that were detected using histochemical and ultrastructural tests.

Keywords: calves, escherichiosis, digestive organs, rennet, intestine, liver, pancreas, histostructure, histochemistry, ultrastructure, morphometry

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К функциональной морфологии органов пищеварения новорожденных телят и патогенезу эшерихиоза

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РЕЗЮМЕ

Несмотря на многочисленные исследования, проблема эшерихиоза новорожденных телят по-прежнему остается одной из актуальных в связи с широким распространением заболевания и высокой смертностью молодняка. В данной статье приводятся результаты экспериментальных исследований, выполненных в ФГБНУ «Всероссийский научно-исследовательский ветеринарный институт патологии, фармакологии и терапии». Целью работы было проведение с помощью современных методов комплексных морфологических исследований для более глубокого понимания вопросов этиологии и патогенеза эшерихиозов. Для этого от 28 телят в возрасте 1–10 сут с установленным диагнозом «колибактериоз» был отобран патологический материал в начальной стадии заболевания, при наличии выраженных клинических признаков и в терминальной стадии болезни. Контролем служил материал от 6 клинически здоровых телят аналогичного возраста. Установлено, что в начальной стадии развития эшерихиоза у новорожденных телят ранние структурные изменения обнаруживались в ультраструктуре клеток слизистой оболочки сычуга и тонкого кишечника, а также в паренхиме печени и слабые – в экзокринной части поджелудочной железы. Наиболее глубокие морфофункциональные изменения наблюдались в органах пищеварения при выраженных клинических признаках заболевания. В этот период развития болезни диапазон патологических процессов расширялся с охватом структурной организации сычуга, тонкого и толстого кишечника, печени и поджелудочной железы. В терминальной стадии эшерихиоза глубокие воспалительные процессы развивались не только в органах пищеварения, но и в других системах организма больных телят. Изменения структуры в органах пищеварения в начальной стадии носили альтеративный характер, тогда как при клинически выраженных признаках болезни они проявлялись в виде катарально-некротического воспаления со множественными кровоизлияниями

в желудочно-кишечном тракте и паренхиматозных органах. В формировании клинико-морфологической картины в начальной стадии развития эшерихиоза у телят ведущее место принадлежало патологии органов пищеварения. В период становления болезни в организме телят развивались изменения на молекулярном и субклеточном уровнях, которые выявлялись гистохимическими и ультраструктурными исследованиями.

Ключевые слова: телята, эшерихиоз, органы пищеварения, сычуг, кишечник, печень, поджелудочная железа, гистоструктура, гистохимия, ультраструктура, морфометрия

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INTRODUCTION

Escherichiosis is common among newborn calves. The infection occurs not only due to the presence of susceptible calves in the holding, but also due to some other factors, such as hypodynamia, keeping adult and young animals together in the same pen, non-compliance with the "all-in all-out" principle, etc. In some farms, the animal disease situation is so severe that nearly all born animals get diseased, and mortality reaches 5–30% and higher [1–9]. In the literature escherichioses are known as dyspepsia, toxic dyspepsia, diarrheal syndrome of infectious etiology (*Escherichia coli*), or colibacteriosis. These diseases cause great economic losses in animal husbandry. The development of a number of pathological conditions occurring only during the neonatal period is determined not only by the age-related anatomical, physiological and immunobiological features of newborn calves, but also by the impact of new environmental conditions on them [1, 4–6, 8, 10–16].

Despite numerous studies of the *E. coli* etiological role in the development of escherichiosis in calves and introduction of mass vaccination, the disease still remains relevant [17–20]. Assessing the scientific achievements in the study of disease issues in young calves, it can be assumed that researchers have discovered many features of escherichiosis etiology and pathogenesis in newborn calves, and also proposed evidence-based recommendations for its control. Following these recommendations makes it possible to significantly reduce the losses of young cattle. However, in general, the problem of morbidity in young cattle, particularly during colostrum feeding period, is far from being solved. A serious disadvantage is that no scientific theory has been developed yet that satisfactorily explains the patterns of this pathology occurrence and development mechanisms in young animals [9, 21–23]. Although the etiological factors of mass escherichiosis of newborn calves are diverse, they are primarily associated with the activation of opportunistic pathogenic microflora, as well as with incompliances with health and hygenic standards for animal keeping and feeding [1, 8, 10, 12], and reduced resistance of young stock against the disease. The escherichiosis morbidity rate in newborn calves in some farms of the Voronezh Oblast (OOO "Voronezhpishcheprodukt") is currently 54% [13].

In this regard, the aim of the research was to conduct comprehensive morphological studies for a deeper understanding of escherichiosis etiology and pathogenesis in newborn calves.

MATERIALS AND METHODS

The study was performed at the FSBSI "All-Russian Veterinary Research Institute of Pathology, Pharmacology and Therapy" (FSBSI "ARVRIPP&T", Voronezh). Samples of pathological material were collected from 28 1–10 day old calves diagnosed with escherichiosis at the initial stage, including those demonstrating apparent clinical signs, and those at the terminal stage of the disease. Samples from 6 clinically healthy calves of the relevant age were used as control.

All experiments were carried out in strict accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS No. 123).

Digestive organ samples were fixed in 10.0–12.0% neutral formalin solution, in Carnoy's and Rossman's liquids for light microscopy, and in 2.5% glutaraldehyde with postfixation in 1.0% osmium tetraoxide solution for electron microscopy. Fresh organ samples were frozen in a cryostat at –20 °C for testing the enzyme activity. The samples were poured into paraffin and Epon-812, followed by the preparation of appropriate sections for light and electron microscopy.

For overview information, the sections were stained with hematoxylin-eosin and hematoxylin-picrofuxin. Histochemical methods were used for detection of the following: lipids – with Sudan III and IV and Sudan black; hemosiderin – according to Perls, mucin – with Meyer's mucicarmine; Golgi complex –according to Elftman;

neutral mucopolysaccharides and glycogen – PAS staining according to Shabadash and Bauer; acid mucopolysaccharides – by alcyan blue staining; ribonucleoproteins (RNP) – by gallocyanin chromium alum staining according to Einarson; RNA – according to Brache; DNA – according to Felgen – Rozenbeck; alkaline phosphatase activity – according to Grokk – Pierce using diazonium salts, acid phosphatase – according to Gomori as modified by Bark; esterase – according to Nahlas – Zeligman; succinate dehydrogenase – using tetrazolium method according to Nachlas, etc.

A single-beam cytophotometer was used for determination of optical density of histochemical preparations in compliance with the manufacturing standards at all stages of the procedure. MOB-1-15$^{\times}$ ocular micrometer (AO "LOMO", Russia) was used for measuring the volume of hepatocyte nuclei, the height of the villi border epithelium and the mucosa thickness.

Hepatocytes, enterocytes and secretory epithelium of the pancreatic exocrine part were studied using ultrastructural methods. For this purpose, electronograms (at least 30 photonegatives at 6,100× magnification from each group of animals) were randomly selected. The volume of cytoplasmic organelles was measured according to the basic rule of stereology [24] using a morphometric grid [25, 26]. The 36-node grid was fixed on the screen of the 5PO-1 type microfilm reader. At the same time, the area of the photonegative (6.5×9 cm) accounted for more than 100 nodes. The number of nodes in the granular (rough) and agranular (smooth) endoplasmic reticula, mitochondria, lipids and hyaloplasm was calculated. After averaging the values for each organelle, the organelle volume was calculated as a percentage of the cytoplasm volume. Confidence intervals of averages were calculated for a confidence level of 0.95.

RESULTS AND DISCUSSION

Initial stage of escherichiosis. At this stage of the disease newborn calves demonstrated early structural changes in the ultrastructure of mucosa cells of the abomasum and small intestine, as well as in the liver parenchyma, and mild changes – in the pancreatic exocrine part.

The abomasum mucosa was swollen with dystrophic changes in the epithelium, and its submucosa contained blood dilated capillaries. There was also an uneven increase and depolymerization of PAS-positive material and acidic mucopolysaccharides. The activity of succinate dehydrogenase slightly decreased in the area of abomasum bottom glands. Single dystrophic mast cells were found in the own plate of mucosa. The ultrastructure of the cylindrical epithelium demonstrated dystrophy of secretory granules and membranes of the endoplasmic reticulum with low activity of nonspecific carboxylic acid esterase (Fig. 1).

Desquamative catarrh of the villi border epithelium was observed in the mucosa of the small intestine, mainly jejunum, where a decreased activity of alkaline phosphatase, succinate dehydrogenase and nonspecific carboxylic acid esterase was noted. At the same time, the activity of acid phosphatase involved in the lysis of cell breakdown products under conditions of dystrophy increased. Vacuolization of cytoplasm, an increase in the number of lysosomes and swelling of mitochondria were observed in the ultrastructure of villi enterocytes.

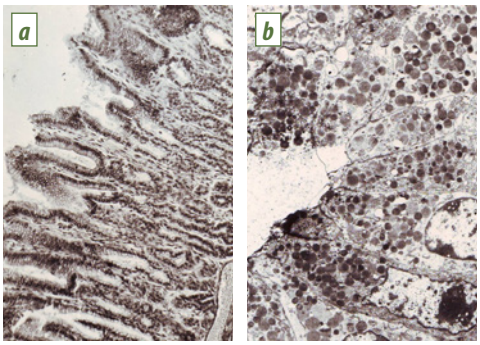

Fig. 1. Structure of rennet mucosa at the initial stage of escherichiosis in calves: a) pyloric glands of a 2-day-old calf in a state of dystrophy; b) degeneration of secretory granules in fundic gland epithelium. Staining with hematoxylin-eosin (a); 8× ocular magnification; 10× objective magnification (a); 6,100× (b)

Small amounts of lymphocytes, histiocytes and mast cells were detected in the own plate of the mucosa villi. Many microvilli were fragmented on the brush border of enterocytes, and demonstrated moderate activity of nonspecific carboxylic acid esterase, succinate dehydrogenase and high activity of acid phosphatase. The RNP amount in the crypts decreased, there was a significant decrease in the villi cells. The number of polymorphic mitochondria with an enlightened matrix increased. The rough endoplasmic reticulum was fragmented and its total volume was 17.7%, and the volume of smooth endoplasmic reticulum increased to 47.0%. Lysosomes were found everywhere. The enterocyte nuclei became hyperchromic. Generally, low secretory activity of enterocytes was detected at the early stage of escherichiosis (Fig. 2).

A moderate amount of glycogen was detected in the liver, weakly expressed fatty and granular dystrophy of hepatocytes and decreased activity of succinate dehydrogenase and nonspecific carboxylic acid esterase were noted. Electron microscopic studies have shown that there was a decrease in the number of glycogen granules in the liver cell cytoplasm. The amount of rough endoplasmic reticulum in hepatocytes decreased to 3.4% as compared to 18.0% in normal. At the same time, the tubules of the endoplasmic reticulum were fragmented and

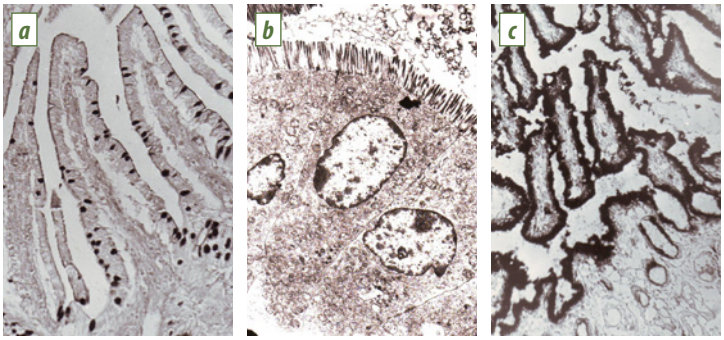

Fig. 2. Histochemical and ultrastructural changes in small intestine mucosa at the initial stage of escherichiosis in calves: a) degranulation of acid mucopolysaccharides in duodenum goblet cells; b) cytoplasm vacuolization and enterocyte microvilli fragmentation; c) irregular activity of succinate dehydrogenase in jejunum. Staining with alcian blue (a); azo-coupling according to Grokk – Pierce (c); 8× ocular magnification; 10× objective magnification (a, c); 6,100× (b)

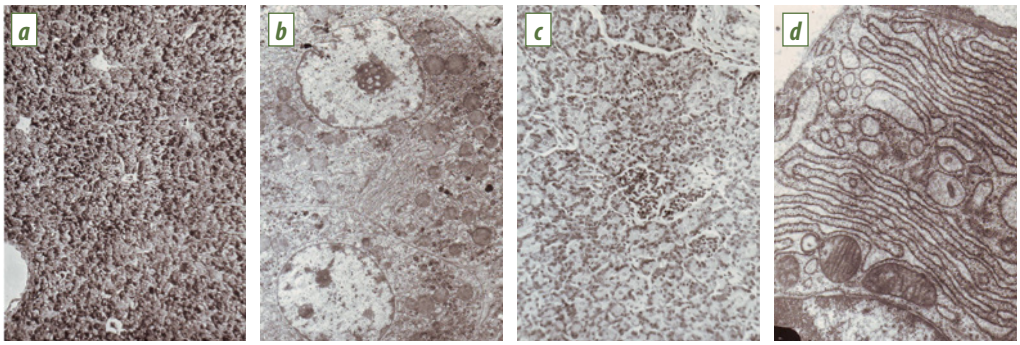

Fig. 3. Histochemical and ultrastructural changes at the initial stage of escherichiosis in calves: a) a moderate amount of glycogen in the liver; b) hepatocyte cytoplasm vacuolization; c) dystrophy of exocrine pancreatic gland acini; d) vacuolization of the acinar cell. Periodic-Acid-Schiff (PAS) staining (a); hematoxylin-eosin staining (c); 8× ocular magnification; 10× objective magnification (a, c); 6,100× (b); 20,000× (d)

turned into large vacuoles. The membranes lost ribosomes. Autophagic vacuoles appeared. Meanwhile, there was an increase in lipid inclusions contacting with mitochondria and endoplasmic reticulum membranes.

No noticeable changes were observed in the pancreas at the initial stage of escherichiosis in calves, but the volume of the granular endoplasmic reticulum and mitochondria in acinar cells significantly decreased, which was 10.0 and 5.0%, respectively (Fig. 3).

In case of pronounced clinical signs of escherichiosis, the most profound morphofunctional changes were observed in the digestive organs. During this period of disease development, the range of pathological processes expanded involving the structural organization of the abomasum, small and large intestines, liver and pancreas.

In the mucous membrane of the abomasum there were foci of epithelial cell desquamation and necrobiosis of the fundal and pyloric gland apex with a sharp decrease in the amount of neutral and acid mucopolysaccharides, nucleoprotein complex and a decrease in the activity of succinate dehydrogenase and nonspecific esterase of carboxylic acids. Conjested blood vessels and submucosa swelling were noted. This led to swelling of the abomasum mucosa, its thickness reached an average of 406.1 microns.

Uncovered villus tips, blood filling and swelling of the own plate were revealed in the small intestine in most cases. The activity of alkaline phosphatase, succinate dehydrogenase and nonspecific carboxylic acid esterase sharply decreased while the acid phosphatase activity in the area of the villi border epithelium increased. Dystrophic changes in enterocytes were accompanied by a significant decrease in the nucleoprotein number, mainly in the villi apical part. The ultrastructure of many villous cells revealed lysis of cytoplasmic organelles with nuclei pyknosis. The apical part of the terminal layer became clearer. Bundles of myofibrils were found in the sites of cell microvilli, and myelin figures and damaged mitochondria appeared in the cytoplasm.

The destruction of the beamed structure was observed in the liver, and hepatocytes were in a state of necrobiosis. Persistent fatty necrobiosis developed in the ultrastructure of hepatocytes, the number of glycogen granules decreased significantly, the membranes of the endoplasmic reticulum and mitochondria were lysed.

Pancreatic acinar cells demonstrating cytoplasm vacuolization and lysis of zymogen granules suffered greatly (Fig. 4).

Terminal stage of escherichiosis. At this stage, deep inflammatory processes developed not only in the digestive organs, but also in other body systems of diseased calves.

Further on, as a rule, the initial damage in the digestive organs progressed due to opportunistic pathogens in the gastrointestinal tract. The damage was characterized by profound changes in the structural organization of cells and tissues with involvement of the large intestine and pancreas in the pathological process. There was a significant decrease in the activity of hydrolytic enzymes and the content of mucopolysaccharide compounds in the gastrointestinal tract and liver. Deeply damaged membrane structures, occurrence of myelin figures, cytolysis and karyolysis were observed in the ultrastructure of abomasum, small intestine and liver mucosa cells. A large number of lipid inclusions accumulated in hepatocytes. At the terminal stage of escherichiosis, microbial intoxication caused irreversible pathological processes in the digestive organs of diseased calves, which in most cases resulted in their death.

CONCLUSION

The digestive organs were structurally and functionally involved in the pathological process in calves at the initial disease stage, as well as those demonstrating clinically pronounced signs of escherichiosis. However, in terms of profoundness of changes and their reversibility, the digestive organs had their own peculiarities. Structural changes in digestive organs had an alterative nature at the initial disease stage, whereas in case of clinically pronounced disease signs there were manifestations of catarrhal-necrotic inflammation with multiple hemorrhages in the gastrointestinal tract and parenchymal organs.

The developing morphological changes corresponded to general pathological processes in newborn calves diseased with escherichiosis. At the same time, a complex of morphological changes characteristic of this pathology developed. The leading role in the formation of the clinical and morphological picture at the initial stage of escherichiosis in calves belonged to the pathology of the digestive organs. As the disease progressed,

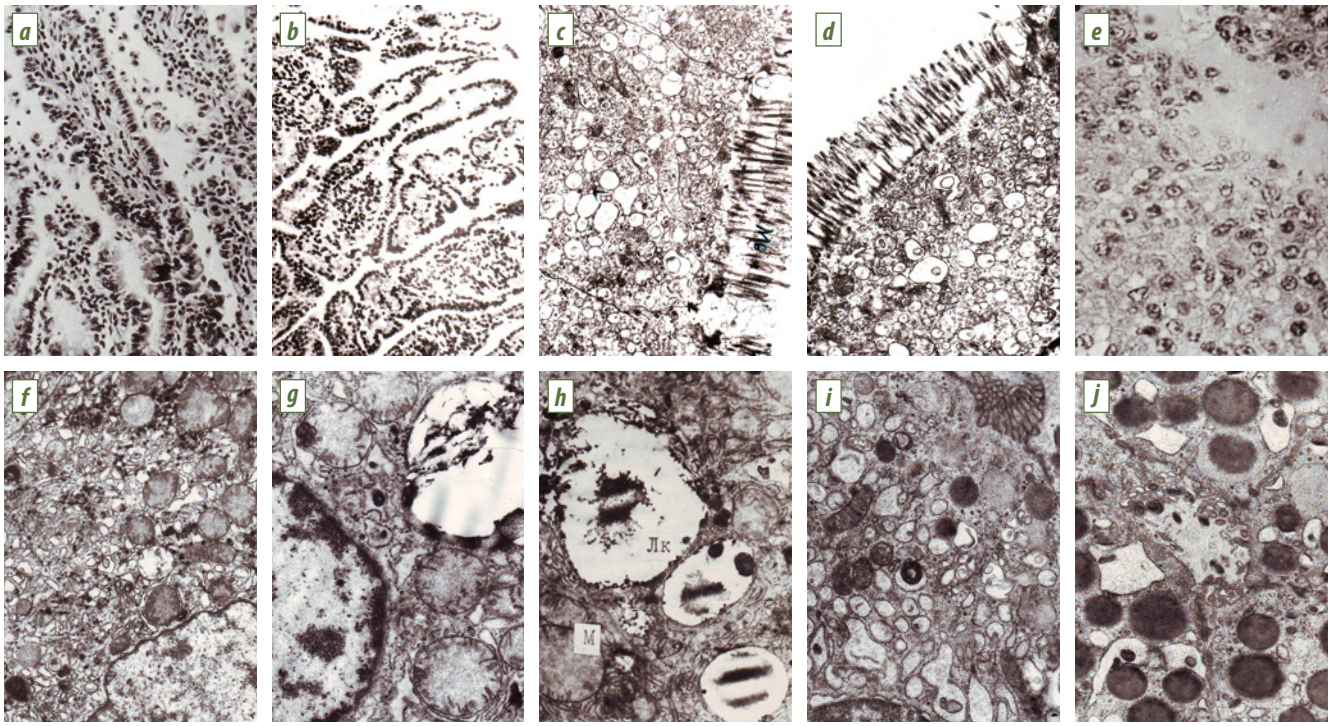

Fig. 4. Structural changes in digestive organs of calves with pronounced clinical signs of escherichiosis: a) desquamative catarrh of the small intestine border epithelium; b) inflammatory-necrotic enteritis; c) lysis of enterocyte cytoplasmic organelles; d) enterocyte necrobiosis and microvilli lysis; e) destruction of the colomnial structure with deep necrobiotic changes in the liver; f) reduction of glycogen granules in hepatocyte cytoplasm and onset of dystrophic changes; g) onset of hepatocyte fatty degeneration; h) progression of hepatocyte fatty necrobiosis; i) vacuolization and absence of zymogen granules in pancreatic acinar cell cytoplasm; j) lysis of microvilli of pancreatic acinar cells and necrobiosis of zymogen granules. Staining with hematoxylin-eosin (a, b, e); 8× ocular magnification; 10× objective magnification (a, b, e); 6,100× (c, d, f); 10,000× (g, h, i, j)

the calves demonstrated changes at molecular and subcellular levels that were detected using histochemical and ultrastructural tests.

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Phylogenetic analysis of dermatophytes isolated from small domestic animals

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SUMMARY

Dermatophytoses are diseases of skin and its accessory structures that are widely spread worldwide. They are most commonly caused by fungi of the genera *Microsporum* and *Trichophyton*. The identification of the agent's species has a great epidemiological significance and is essential for effective therapy. The aim of the study is the identification and phylogenetic analysis of dermatophytes isolated from dogs and cats in the Republic of Kazakhstan and the Russian Federation by means of molecular techniques. The fungal isolate species were confirmed by sequencing using two rDNA internal transcribed spacer (ITS) primer pairs, and this allowed for their deposition to the GenBank database. Based on the sequencing results, *Microsporum canis* (12 strains) and *Trichophyton benhamiae* (2 strains) were identified. The nucleotide sequences were analysed, and phylogenetic trees were constructed, taking into account the results of the dermatophyte identification using two primer pairs. The constructed phylogenetic trees reflecting the relationships of dermatophytes showed that, irrespective of the primer pairs used, the *Microsporum* and *Trichophyton* pathogens are in all cases reliably assigned to different clades. The analysis of *ITS4F/ITS5R* sequence fragment structures enabled the establishment of genetic relatedness between the *Trichophyton benhamiae* strains first isolated from cats in Russia and the Russian strain recovered from a guinea pig. The comparative analysis of the genomes of the *Microsporum* and *Trichophyton* fungi and reference strains revealed a relatively low level of intraspecies polymorphism and point mutations of the sequences. The data analysis demonstrated a high percentage of nucleotide sequence homology, and this allows using the primers for PCR tests intended for dermatophytosis diagnosis in cats and dogs.

Keywords: dermatophytosis, *Microsporum canis*, *Trichophyton benhamiae*, phylogenetic tree, homology, reference strain, nucleotide sequence

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Филогенетический анализ дерматофитов, выделенных от мелких домашних животных

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РЕЗЮМЕ

Дерматофитозы – широко распространенные во всем мире заболевания кожи и ее производных, чаще всего вызываемые грибами родов *Microsporum* и *Trichophyton*. Идентификация вида возбудителя имеет большое эпидемиологическое значение, а также необходима для проведения эффективной терапии. Цель исследований – идентификация и филогенетический анализ дерматофитов, выделенных от собак и кошек на территории Республики Казахстан и Российской Федерации, с помощью молекулярных методов. Видовая принадлежность изолятов грибов была подтверждена секвенированием по двум парам праймеров внутреннего транскрибируемого спейсерного участка (internal transcribed spacer, ITS) рДНК, что позволило депонировать их в базу данных GenBank. На основании результатов секвенирования были идентифицированы *Microsporum canis* (12 штаммов) и *Trichophyton benhamiae* (2 штамма). Проведен анализ нуклеотидных последовательностей и построены филогенетические деревья с учетом результатов идентификации дерматофитов по двум парам праймеров. Построение филогенетического дерева, основанное на отражении родственных связей дерматофитов, показало, что, независимо от использования разных пар праймеров, возбудители рода *Microsporum* и *Trichophyton* во всех случаях достоверно распределены по разным кладам. Анализ структур фрагментов последовательности *ITS4F/ITS5R* позволил выявить генетическое родство штаммов *Trichophyton benhamiae*, впервые выделенных от кошек на территории России, с российским штаммом, изолированным от морской свинки. Сравнительный анализ геномов грибов рода *Microsporum* и *Trichophyton* с референтными штаммами показал относительно невысокий уровень внутривидового полиморфизма и точечных мутаций

последовательностей. В результате анализа данных был определен высокий процент гомологии нуклеотидных последовательностей, что позволяет использовать праймеры для проведения полимеразной цепной реакции в качестве диагностического теста при дерматофитозах кошек и собак.

Ключевые слова: дерматофитоз, *Microsporum canis*, *Trichophyton benhamiae*, филогенетическое дерево, гомология, референтный штамм, нуклеотидная последовательность

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INTRODUCTION

Dermatophytes are keratinophilic fungi of the family *Arthrodermataceae* (*Onygenales*, *Ascomycota*) that include dozens of related species differentiated mainly by their anamorphs or asexual forms and arranged in three classical genera: *Trichophyton*, *Microsporum* and *Epidermophyton* [1]. The genera *Trichophyton* and *Microsporum* comprise anthropophilic, zoophilic and geophilic dermatophyte species that are able to cause infection mostly in humans or animals and found free-living in soil. The genus *Epidermophyton* includes only one species, *E. floccosum*, which exclusively affects humans.

Dermatophytoses, diseases caused by these fungi, are spread worldwide, with the number of cases in humans and animals increasing annually. The occurrence of dermatophytoses in small domestic animals such as cats and dogs being the companion animals of humans is of particular significance [2–3]. *Microsporum canis* and *Trichophyton mentagrophytes* are the most significant species of dermatophytes isolated from infected dogs, cats and other carnivores [5, 6].

Until recently, the diagnosis of dermatophytoses has been based on the clinical signs of the disease, which are unreliable due to the variable nature of dermatological lesions and similarity with other skin diseases that mimic the symptoms characteristic of dermatophytoses [6]. The direct microscopic examination of biological material samples collected from lesions and the isolation of dermatophyte cultures in nutrient media are the gold standard for dermatophytosis diagnosis. However, species identification may sometimes require further investigation of the biochemical properties of the isolated dermatophyte cultures. Therefore, dermatophyte species identification based on studying their phenotypic characteristics is a labour-intensive and time-consuming process that requires skilled researchers [7].

Molecular techniques are promising for the direct detection of fungal DNAs in the clinical samples and their species identification [8]. At present, methods based on ribosomal gene nucleotide sequencing are utilized for dermatophyte species identification in some countries [9]. Data on fully or partially sequenced rRNA genes of various microorganisms are submitted to the international databases and can be used as reference ones. The comparative analysis of sequences of genes and individual gene regions encoding ribosomal RNAs may contribute to the detection of dermatophyte relationships [10]. Multilocus microsatellite typing was applied for tracking the routes of spread and transmission of *M. canis* in Japan [11]. The findings from a study on *M. canis* occurrence in cats, dogs and humans by means of molecular genetic typing using forward (*ITS1* 5′-TC CGTAGGTGAACCTGCGG-3′) and reverse (*ITS4* 5′-TCCTCCGCTTATTGATATGC-3′) primers showed that indoor and outdoor animals, as well as cats and dogs with or without the disease symptoms are the main dermatophyte sources for humans [12]. The application of molecular techniques allowed for the determination of the etiological structure of dermatophytoses in Iran, which was represented by the following species: *M. canis* – 78.5%, *M. gypseum* – 10.7% and *T. mentagrophytes* – 10.7% [13].

The study was aimed at the assessment of the possibility of using molecular techniques for the identification and phylogenetic analysis of dermatophytes isolated from dogs and cats in the Republic of Kazakhstan and the Russian Federation.

MATERIALS AND METHODS

The objects of the study were the intergenic internal transcribed spacer (*ITS*) region 5.8, 18, 28S rRNA nucleotide sequences of the representatives of two dermatophyte genera, *Microsporum* ($n = 12$) and *Trichophyton* ($n = 2$), isolated from dogs and cats in the Republic of Kazakhstan and the Russian Federation (Table).

The amplification of marker genes (*ITS*) was carried out in the final reaction volume of 25 µL containing 1× Phusion HF-buffer, 2.5 mM of $MgCl_2$, 1U Phusion DNA-polymerase and 200 µM of dNTP (New England BioLabs Inc., USA), 25 pmol of each primer and 20 ng of extracted DNA from one sample.

Thermal cycling conditions for polymerase chain reaction (PCR) were as follows: initial DNA denaturation at 95 °C for 5 minutes, then 35 cycles at 95 °C for 30 seconds,

at 58 °C for 40 seconds, at 72 °C for 50 seconds and final extension at 72 °C for 5 minutes. The amplified DNA products were analysed with horizontal 1.5% agarose gel electrophoresis using 1× TAE buffer and EtBr. The electrophoresis parameters were 120 V, 250 mA, 50 W, the reaction time was 30 minutes. The amplified DNA fragments were sequenced using the Sanger method and a BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA).

The primer sequences used were the same as for PCR.

To ensure the accuracy of results, the amplified fragments were sequenced using two primer pairs: the forward primer *ITS1F* (TCCGTAGGTGAACCTGCGG) and the reverse primer *ITS4R* (TCCTCCGCTTATTGATATGC) [12]; the forward primer *ITS4F* (TCCTCCGCTTATTGATATGC) and the reverse primer *ITS5R* (GGAAGTAAAAGTCGTAACAAGG) [14].

The sequencing products were analysed using an ABI 3130XL Genetic Analyzer (Applied Biosystems, USA). The chromatograms were analysed and edited using Sequencing Analysis Software v5.2, Patch 2 (Applied Biosystems, USA). The resulting sequences were deposited to the international GenBank database. The multiple sequence alignment was carried out using MUSCLE and ClustalW algorithms.

The phylogenetic analysis was performed using the maximum likelihood and nearest neighbour methods, as well as MEGA (v11) bioinformatic analysis software.

RESULTS AND DISCUSSION

M. canis and *T. benhamiae,* the agents of dermatophytoses in cats and dogs, were identified based on the results of sequencing by the Sanger method using the primer pair *ITS1F/ITS4R* (12 *Microsporum* and 2 *Trichophyton* isolates) and the primer pair *ITS4F/ITS5R* (6 *Microsporum* isolates and 2 *Trichophyton* strains).

The analysed isolates showed a high percentage of nucleotide sequence homology with reference strains, and this allowed for their deposition to the international NCBI database. The following unique identifiers were assigned based on the *ITS1F/ITS4R* genotyping results: *M. canis* – OQ592853.1, OQ592883.1, OQ592896.1, OQ592901.1, OQ593382.1, OQ593383.1, OQ593387.1, OQ593395.1, OQ593394.1, OQ594023.1, OQ594046.1, OQ594324.1; *T. benhamiae* – OQ592797.1, OQ600605.1.

The following unique identifiers were assigned based on the *ITS4F/ITS5R* genotyping results: *M. canis* – ON527772.1, ON527773.1, ON527774.1, ON527775.1,

Table
The list of dermatophyte isolates recovered from dogs and cats

No.	Suspected pathogen	Isolate No.	Date of isolation in nutrient media	Animal species	Region
1	*Microsporum* spp.	5	04.05.2021	female cat	Republic of Kazakhstan
2		8	31.05.2021		
3		22	14.09.2021		
4		27	17.09.2021		
5		29	21.09.2021	male cat	
6		33	10.10.2021		
7		35	11.10.2021		
8		48	28.10.2021	female cat	
9		58	21.12.2021		
10		61	25.12.2021	dog	
11		64	25.12.2021		
12	*Trichophyton* spp.	68	12.01.2022	female cat	
13	*Trichophyton* spp.	19	02.12.2021	female cat	Russian Federation
14		20	02.12.2021	male cat	

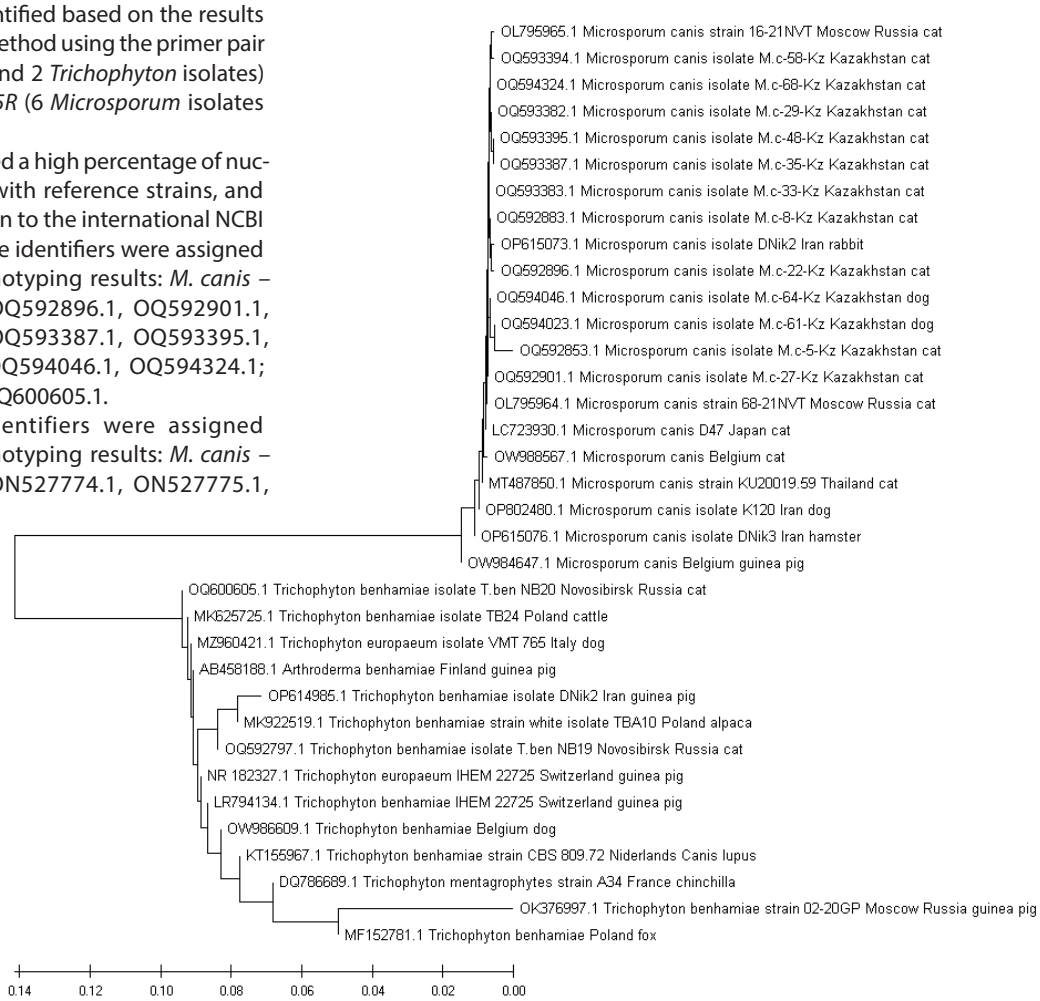

Fig. 1. Phylogenetic tree of Microsporum and Trichophyton spp. dermatophytes based on ITS1F/ITS4R sequence fragment structure analysis

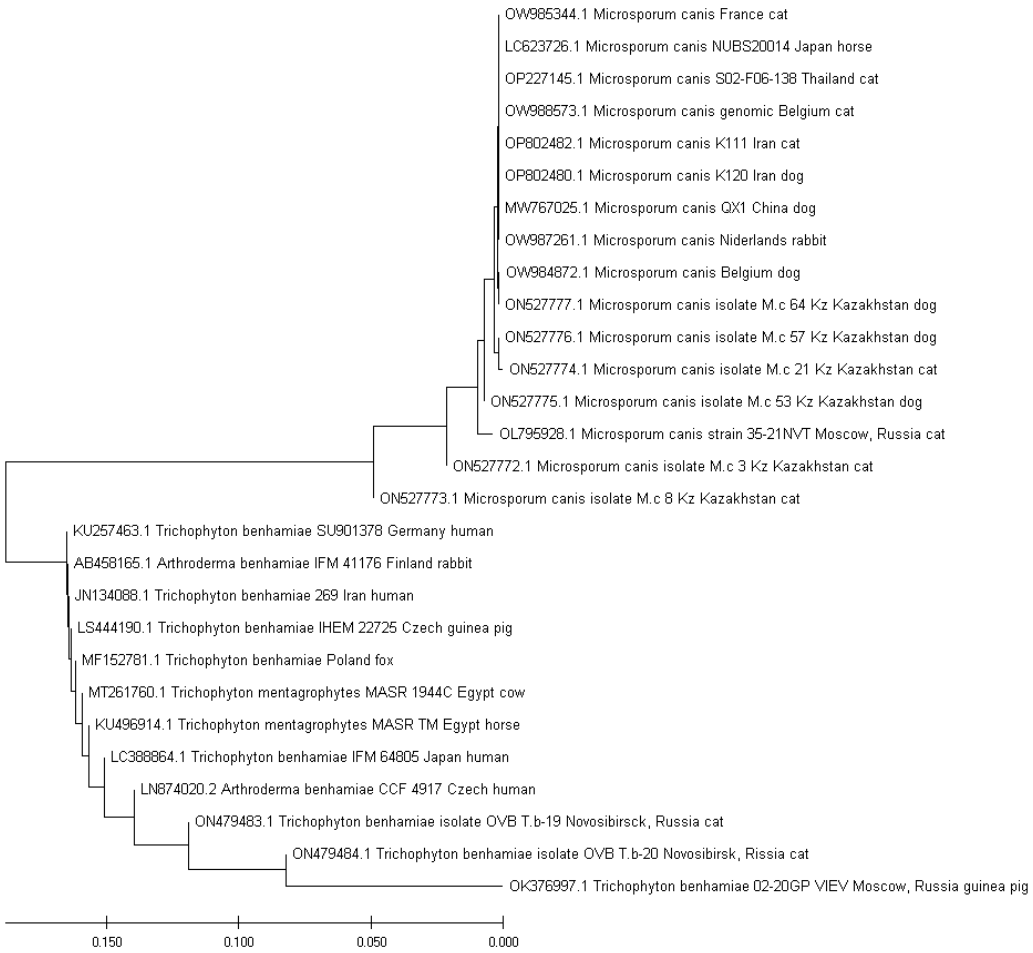

Fig. 2. Phylogenetic tree of Microsporum and Trichophyton spp. dermatophytes based on ITS4F/ITS5R sequence fragment structure analysis

ON527776.1, ON527777.1; *T. benhamiae* – ON479483.1, ON479484.1.

To construct a phylogenetic tree, the database of complete intergenic region 5.8, 18, 28S rRNA nucleotide sequences was searched for the typical representatives of *Microsporum* and *Trichophyton* dermatophytes to be used as reference strains for the comparative phylogenetic analysis of the identified strains.

The evolutionary relationship of the strains was inferred using the neighbour-joining method [15]. The phylogenetic tree was drawn to scale, with the evolutionary distance corresponding to 14 substitutions per 100 nucleotides. The evolutionary distances were computed using the maximum composite likelihood method [16] and expressed in the units of the number of base substitutions per site. All ambiguous positions were removed for each sequence pair (the pairwise-deletion option). There was a total of 1,419 positions in the final dataset. The phylogenetic trees based on the *ITS1F/ITS4R* and *ITS4F/ITS5R* sequence fragment structure analysis and reflecting the relationships of *Microsporum* and *Trichophyton* dermatophytes are presented in Figures 1 and 2, respectively.

The optimal tree presented in Figure 2 is drawn to scale, with the evolutionary distance corresponding to 15–20 substitutions per 100 nucleotides. The analysis included 28 nucleotide sequences. All ambiguous positions were removed for each sequence pair (the pairwise-deletion option). There was a total of 1,152 positions in the final dataset. The evolutionary analysis was carried out using MEGA (v11) bioinformatic analysis software [17].

The constructed phylogenetic tree reflecting the relationships of dermatophytes showed that, irrespective of the primer pairs used, the *Microsporum* and *Trichophyton* pathogens are in all cases reliably assigned to different clades. The analysis of *ITS4F/ITS5R* sequence fragment structures enabled the establishment of genetic relatedness between all *T. benhamiae* strains first isolated from cats in Russia and the Russian strain recovered from a guinea pig.

The evolutionary relationship of the strains inferred by the analysis of the genome data for each dermatophyte strain obtained using the primers *ITS1F/ITS4R* is shown in Figures 3 and 4.

Sequences used for comparison of the relevant *M. canis* strain intergenic region rDNA sequence were selected from those of the outgroup strains.

Figure 3 shows the high homology of *M. canis* strain intergenic region 5.8, 18, 28S rDNA sequences. The LC723930.1 and MT487850.1 sequences have only one nucleotide substitution each, which is indicative of point mutation. OL795964.1 was found to have three mononucleotide deletions (i.e. polymorphism). The OW988567.1 sequence and that of the Kazakhstan OQ594324.1 strain (marked with an asterisk *) were found to have only one deletion.

Similar methods were used for the analysis of the relevant *T. benhamiae* strain intergenic region rDNA sequence.

Data presented in Figure 4 show that the OQ592797.1 strain sequence (marked with an asterisk *) has additional nucleotide insertions indicative of polymorphism. The second *T. benhamiae* strain, OQ600605.1, was homologous to the reference strains. Point mutations were detected in the OP614985.1, MK922519.1, MK625725.1 strain sequences. There were two substitutions in the OK376997.1 strain sequence, and this is also indicative of point mutations.

The evolutionary relationship of the strains inferred by the analysis of the genome data for each dermatophyte strain obtained using the primers *ITS4F/ITS5R* is shown in Figures 5 and 6.

Sequences used for comparison of the relevant *M. canis* strain intergenic region rDNA sequence were selected from those of the outgroup strains.

Figure 5 shows that nine out of eleven sequences presented have a single polymorphism, one sequence has a mutation. The analysed sequence (marked with an asterisk *) has three nucleotide substitutions.

The comparative analysis of the relevant *T. benhamiae* strain intergenic region rDNA sequences using the primers *ITS4F/ITS5R* is presented in Figure 6.

As we can see, all the nucleotide sequences of the *T. benhamiae* strains are homologous. The sequences of three strains (MT261760.1, KU496914.1 and OK376997.1) have one mutation each. *T. mentagrophytes* MT261760.1 and KU496914.1 strains were found to have two nucleotide deletions. These strains belong to the same clade and are not the members of the species *T. benhamiae*, and this reliably increases the specificity of the diagnostic test and enables differentiation between the representatives of two species of the same genus.

OK376997.1 strain is the representative of the species *T. benhamiae* isolated from a guinea pig. In comparison with the analysed T. *benhamiae* strains isolated from cats, its nucleotide sequences have one difference, namely a point mutation.

In view of these findings, we would emphasize the importance of dermatophytosis pathogen species identification. This is particularly important for the species *T. benhamiae,* a new causative agent of dermatophytosis in cats, first isolated by us in Russia [18].

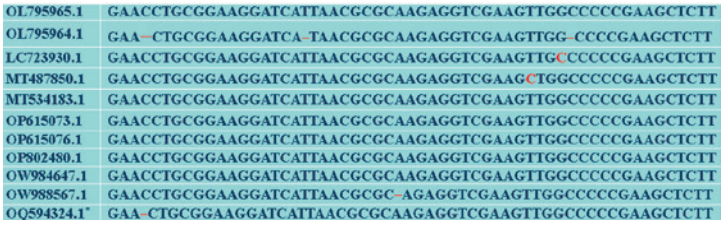

Fig. 3. M. canis strain intergenic region 5.8, 18, 28S rDNA nucleotide sequence sites containing nucleotide substitutions

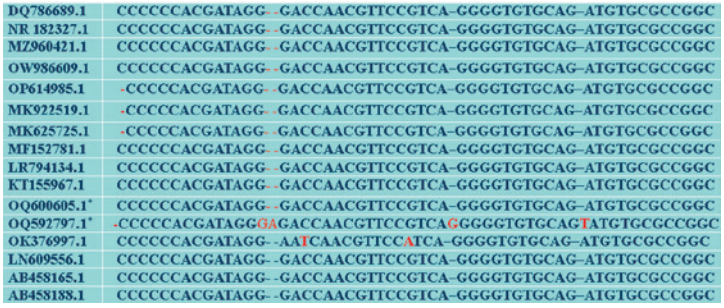

Fig. 4. T. benhamiae strain intergenic region 5.8, 18, 28S rDNA nucleotide sequence sites containing nucleotide substitutions

Fig. 5. M. canis strain intergenic region 5.8, 18, 28S rDNA nucleotide sequence sites containing nucleotide substitutions

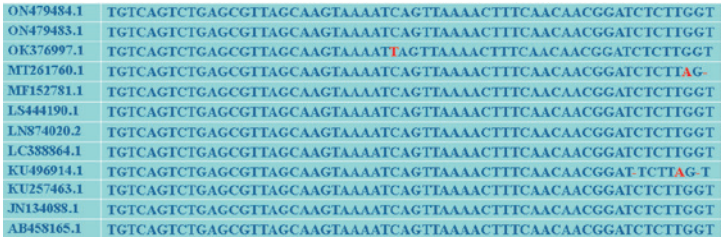

Fig. 6. Trichophyton spp. strain intergenic region 5.8, 18, 28S rDNA nucleotide sequence sites containing nucleotide substitutions

CONCLUSION

The phylogenetic analysis of 12 *Microsporum* and 2 *Trichophyton* strains demonstrated a high percentage of their nucleotide sequence homology. The comparative analysis of the ribosomal RNA gene fragments of the *Microsporum* and *Trichophyton* fungi and reference strains revealed in each case a relatively low level of intraspecies polymorphism and point mutations of the sequences. This shows that *ITS*-region 5.8, 18, 28S rDNA gene nucleotide sequencing can be considered as a rapid and reliable technique for the identification of closely related dermatophytes of the genera *Trichophyton* and *Microsporum.* The detected similarity of the nucleotide sequences of the analysed and reference strains of dermatophytes is indicative of the reliability of the results obtained and the possibility of using molecular diagnostic techniques for their species identification.

Thus, *ITS*-PCR is a reliable and robust method for the identification of closely related dermatophyte species and can therefore be used for dermatophytosis diagnosis in cats and dogs.

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Remuneration of veterinary specialists of the State Veterinary Service in the Russian Federation Subjects

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SUMMARY

Remuneration is one of the main factors that can motivate an employee to work productively, as well as influence the prestige of a profession. The paper presents the results of analysis of remuneration of the veterinary specialists of the State Veterinary Service for the country as a whole, for the federal districts and 85 regions of the country, as well as at various organizational levels within the Veterinary Service for 2021. The assessment of the veterinary specialist salary level as compared with that throughout the economy of the Russian Federation Subjects is presented. The veterinary specialist average monthly salary for the State Veterinary Service as a whole varies within a wide range: from 16.3 to 114.9 thousand rubles. The minimum and maximum salaries at different organizational levels within the State Veterinary Service differ 5–8-fold. The veterinary specialist salary level is lower than that for the economy of the relevant Russian Federation Subject: for the State Veterinary Service as a whole – in 69 regions of the country, for treatment and preventive care institutions – in 71 Subjects, in laboratory diagnosis institutions – in 72 Subjects. Of all the federal districts, the North Caucasian Federal District has the lowest veterinary specialist salary level both in absolute terms (about 21 thousand rubles) and as compared with the average for the economy of the Subjects of this federal district (64%). The paper also examines veterinary specialist modal and median salary values, which allow for the assessment of remuneration of this category of the State Veterinary Service staff from different viewpoints.

Keywords: remuneration, funding of the Veterinary Service, veterinary specialist, Veterinary Service, organizational levels within the Veterinary Service, median salary, modal salary

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Оплата труда ветеринарных специалистов государственной ветеринарной службы в субъектах Российской Федерации

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РЕЗЮМЕ

Одним из главных факторов, способных мотивировать работника к продуктивному труду, а также влиять на престиж профессии, является уровень заработной платы. В статье представлены результаты анализа заработной платы ветеринарных специалистов государственной ветеринарной службы в целом по стране, по федеральным округам, по 85 регионам страны, а также на различных уровнях организации ветеринарной службы в 2021 г. Приведена оценка заработной платы ветспециалистов в сравнении с зарплатой, сложившейся в целом по экономике субъектов Российской Федерации. Размер среднемесячной заработной платы ветеринарных специалистов в целом по госветслужбе варьируется в широком диапазоне: от 16,3 до 114,9 тыс. руб. На разных уровнях организации госветслужбы отличия между минимальной и максимальной заработной платой составляют 5–8 раз. Размер оплаты труда ветспециалистов не достигает размера оплаты труда, сложившегося по экономике своих субъектов, в целом по госветслужбе – в 69 регионах страны, в учреждениях лечебно-профилактического звена – в 71 субъекте, в организациях лабораторно-диагностического профиля – в 72 субъектах. Из всех федеральных округов наибольшее отставание в заработной плате отмечается в Северо-Кавказском федеральном округе как в абсолютном значении (около 21 тыс. руб.), так и по отношению к средней по экономике субъектов данного федерального округа (64%). В работе рассмотрены также значения модальной и медианной заработных плат ветеринарных специалистов, позволяющие с разных сторон оценить размер оплаты труда данной категории сотрудников госветслужбы.

Ключевые слова: оплата труда, финансирование ветеринарной службы, ветеринарный специалист, ветеринарная служба, уровни организации ветеринарной службы, медианная зарплата, модальная зарплата

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INTRODUCTION

A veterinary specialist is one of the highly demanded professions. The effective organization of the Veterinary Service of the country is essential for ensuring both animal and human health, since certain animal diseases also pose a risk for humans. Besides, animal health professionals play an important role in the food security of the country.

The performance of the State Veterinary Service is influenced by many factors, with remuneration being one of the key ones. The salary depends on the location, the budget of a region, the size/level of a veterinary institution, a veterinary specialist's qualification, etc. In most cases, remuneration is the main motivation for employees to properly fulfill their responsibilities, with an impact also on the availability of qualified personnel in this sphere [1]. At the same time, veterinary specialist remuneration does not always match the complexity and significance of the work performed and the responsibility incurred. Active measures are, therefore, required to improve the veterinary specialist remuneration system to ensure the preservation and development of human resources. Decent salaries for this category of staff constitute the basis for the appropriate implementation of tasks assigned and the enhancement of the veterinary profession prestige [2]. Particularly given that the improvement of the living standards and the reduction of imbalances between the regions of the country are among the main directions of the social policy of the Government [3, 4].

The aim of the study was to assess the veterinary specialist salary level for the State Veterinary Service as a whole, for the Russian Federation Subjects and at different organizational levels within the State Veterinary Service.

MATERIALS AND METHODS

Data for 2021 obtained from the veterinary executive authorities of the Russian Federation Subjects served as a practical basis for the analysis of remuneration of the veterinary specialists of the State Veterinary Service of the Russian Federation Subjects. The data were provided according to the primary data collection form developed by the FGBI "ARRIAH" and covered the following organizational levels within the Veterinary Service:

– veterinary executive authorities of the Russian Federation Subjects (departments, committees, administrations, etc.), exclusive of their subordinate institutions;

– treatment and preventive care institutions (animal disease control stations, veterinary centres, veterinary clinics, veterinary units, veterinary offices, etc.);

– laboratory diagnosis institutions (veterinary diagnostic laboratories of different levels: oblast, raion, inter-raion, etc.).

The information was submitted from 85 Subjects of the Russian Federation. The data were collected using the "Assol.Express" component of the Federal Governmental Information System "VetIS" (FGIS "VetIS").

The theoretical and methodological framework for the study included the laws and regulations of the Russian Federation, data from the Federal State Statistics Service (Rosstat) and analysis methods described in the contemporary scientific papers on this subject.

Analysis techniques, the methods of comparative analysis, descriptive statistics, consolidation, grouping, graphing were used for the study.

RESULTS AND DISCUSSION

The activities of the State Veterinary Service of the Russian Federation Subjects were funded from three sources: the federal budget, the budgets of the Subjects and extra-budgetary sources [5, 6]. The funds allocated for salaries averaged 65% of the total funding in this sphere. The funds for the payment of staff salaries were allocated as follows: 2% from the federal budget (only 12 Subjects received the funding), 61% from the budgets of the Russian Federation Subjects and 37% from the extra-budgetary sources of the veterinary institutions of the State Veterinary Service of the regions.

The percentage of funds allocated for salaries varied from Subject to Subject, ranging from 26 to 86% of the total funding of the regional Veterinary Service. However, based on the analysis results, there is no direct correlation between this indicator and the veterinary specialist salary level (both in absolute terms and as compared with the average for the economy of a particular Subject). Everything depends on the actual amount of the Veterinary Service funding in a particular region of the country.

Figure 1 presents the veterinary specialist average monthly salary for the State Veterinary Service as a whole and at different organizational levels within the Veterinary Service.

The data show that the veterinary specialist average monthly salary for the State Veterinary Service as a whole

varies within in a wide range from 16.3 to 114.9 thousand rubles from Subject to Subject (42 thousand rubles on average). Salary values differ 7-fold for the treatment and preventive care institutions, 5-fold for the laboratory diagnosis institutions and 8-fold for the veterinary executive authorities.

There is also a great deal of remuneration variation across the federal districts. For the Veterinary Service as a whole, it ranged from 21 thousand rubles in the North Caucasian Federal District to 64 thousand rubles in the Far Eastern Federal District (Fig. 2). High incomes are most commonly reported for the Subjects, in which northern allowances and other compensations are established, as well as for Moscow. It is not entirely correct to compare these regions with many others; therefore, a relative indicator representing the ratio of the veterinary specialist salary in a Russian Federation Subject to that throughout the economy of this Subject will be more significant (Fig. 3).

The diagram shows that the veterinary specialist average salary for the Veterinary Service as a whole is lower than the average monthly nominal salary for the economy of a particular Subject in 69 out of 85 regions of the country; among these, the veterinary specialist average salary in 24 Subjects amounted to 81 to 90% of that for the relevant region. The veterinary specialist salary exceeded the average for a Subject only in 16 regions of the country. In 2 Subjects, the veterinary specialist average salary made up only 41 and 44% of the average for the economy of these Subjects.

The veterinary specialist salary in the veterinary treatment and preventive care institutions in more than half of the country's regions (namely, in 46) ranged from 71 to 90% of the average monthly salary for the economy of the relevant Subject (Fig. 4). The salary was close to the regional level in 9 Subjects and exceeded it in 14 Subjects. The largest salary gap was observed in 16 Subjects of the country, where the veterinary specialist salary was 30–50% lower than the average for the relevant regions.

The veterinary specialist salary in the veterinary diagnostic laboratories in 19 regions of the country ranged from 71 to 80% of the average monthly salary for the economy of these Subjects (Fig. 5). The salary was below this level in 28 Subjects; in 5 Subjects out of these, it made up only 34–44% of the average for the relevant Subject. The situation appears to be most optimistic only in 10 Russian Federation Subjects.

The veterinary executive authority staff salary was lower than the average nominal salary for the relevant Subject only in 5 Subjects and exceeded it in other regions.

The domestic statistics mainly relies on the average salary. However, due to large income differences, it does not accurately reflect the real salary level. Therefore, the comparative assessment of the average salary and the most common (modal) / middle (median) salaries is of particular interest [7, 8]. Let us consider these salary values for the veterinary specialists of the State Veterinary Service and compare them with the above-mentioned average values.

A modal salary is the most frequently occurring salary value for a particular category of staff, here – for veterinary specialists [9]. Veterinary specialist salary distribution based on factual data for 85 Russian Federation Subjects is shown in Figure 6.

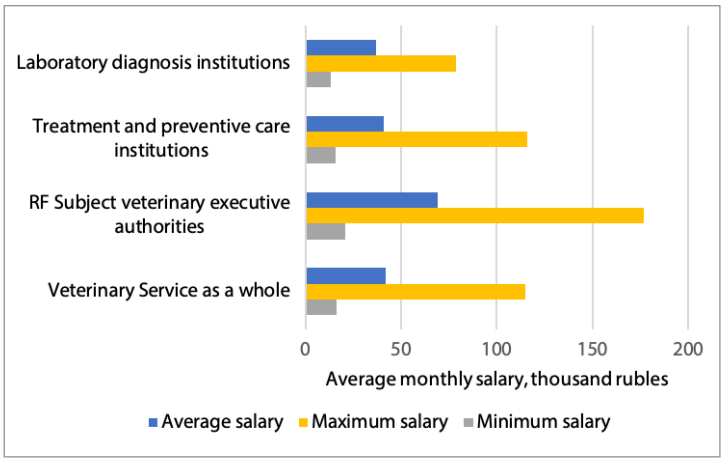


Fig. 1. Veterinary specialist remuneration in the Russian Federation by organizational level within the Veterinary Service, thousand rubles

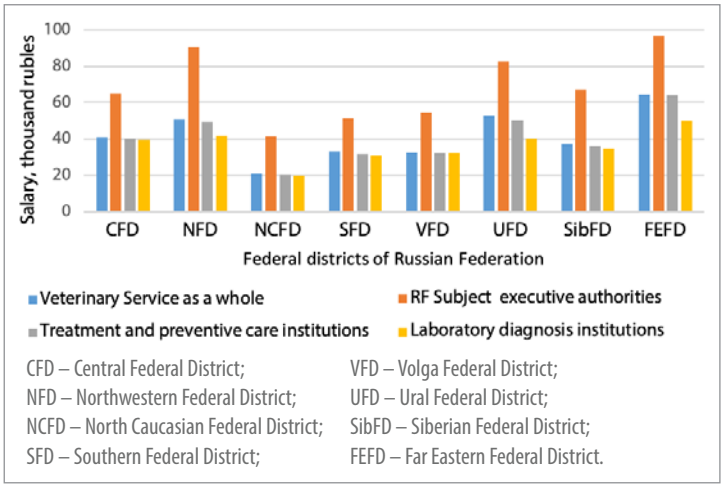


Fig. 2. Veterinary specialist remuneration in the federal districts of the Russian Federation, thousand rubles

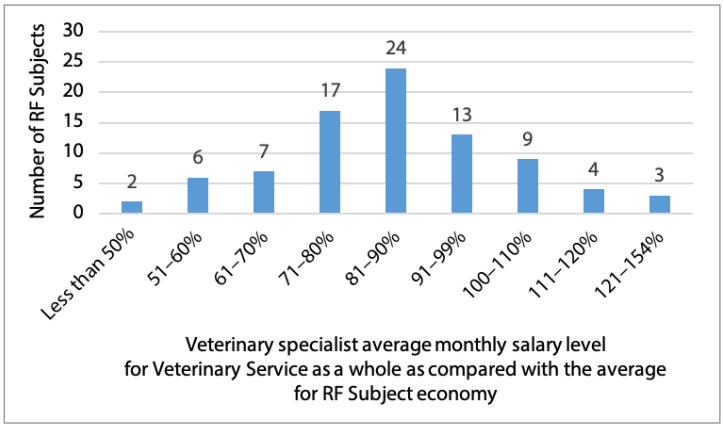


Fig. 3. Veterinary specialist salary level for the State Veterinary Service as a whole as compared with the average for the Russian Federation Subject economy

Based on the data presented, the veterinary specialist modal salary in Russia for the Veterinary Service as a whole ranged from 35 to 40 thousand rubles; that for the veterinary executive authorities – from 50 to 55 thousand rubles; for the treatment and preventive care institutions – from

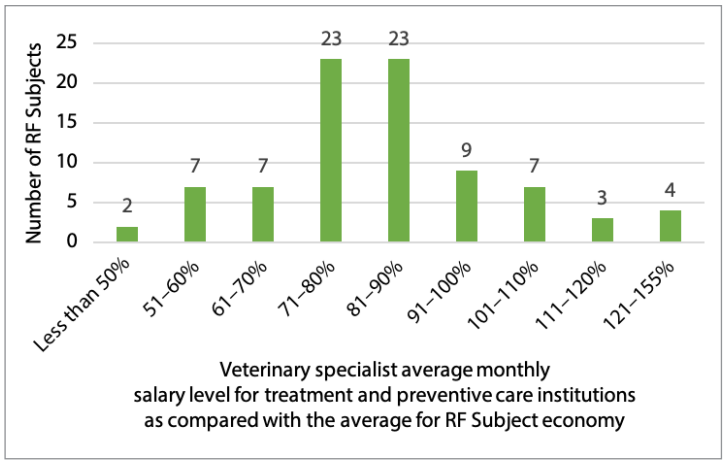


Fig. 4. Veterinary specialist salary level for treatment and preventive care institutions as compared with the average for the Russian Federation Subject economy

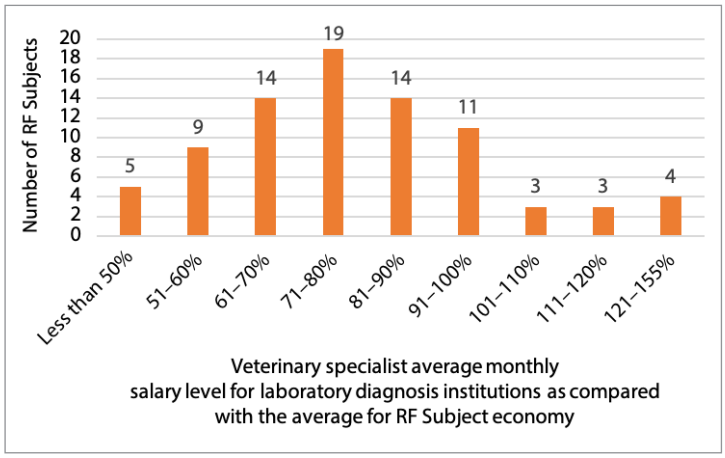


Fig. 5. Veterinary specialist salary level for laboratory diagnosis institutions as compared with the average for the Russian Federation Subject economy

35 to 40 thousand rubles; for the laboratory diagnosis institutions – from 25 to 30 thousand rubles.

For intervals with the highest frequency (18, 13, 20 and 19, respectively), the conditional mode value was calculated using the formula:

$$Mo = X_{Mo} + i_{Mo} \frac{f_{Mo} - f_{Mo-1}}{2f_{Mo} - f_{Mo-1} - f_{Mo+1}},$$

where Mo is the mode;

X_{Mo} is the lower (initial) limit of the modal interval;

i_{Mo} is the size of the modal interval (difference between its upper and lower limits);

f_{Mo} is the frequency of the modal interval;

f_{Mo-1} is the frequency of the interval preceding the modal one;

f_{Mo+1} is the frequency of the interval succeeding the modal one.

Based on the calculation results for each organizational level within the Veterinary Service, the veterinary specialist modal salary for the State Veterinary Service of the Russian Federation as a whole was about 36 thousand rubles; for the executive authorities – 54 thousand rubles; for the treatment and preventive care institutions – 35 thousand rubles; for the laboratory diagnosis institutions – 28 thousand rubles, i.e. somewhat lower than the above arithmetic mean values.

The modal salary reflects the most common salary value for this category of specialists, and the median salary is the middle of the statistical series of all salaries under consideration for 85 Russian Federation Subjects. The median salary indicates that the veterinary specialists of the State Veterinary Service in 50% of the Russian Federation Subjects earn more than this value and those in the other 50% of the Subjects earn less than this value [10, 11].

The veterinary specialist salary level for the Veterinary Service as a whole ranged from 16.3 (n = 1) to 114.9 (n = 85) thousand rubles across the Russian Federation Subjects, and the median value was 37.0 thousand rubles (n = 43) (n is the sequential number of a Subject in the ranked set of salary values). This means that the veterinary specialist salary for the State Veterinary Service as a whole is less than 37.0 thousand rubles in 42 Subjects of the country. This value for the executive authorities is about 58 thousand rubles; for the treatment and preventive care institutions – 36 thousand rubles; for the laboratory diagnosis institutions – 35 thousand rubles.

It should be noted that the median salary serves now as an important indicator for the determination of the minimum salary level in the country, which does not depend on the minimum subsistence level of the working population, as was the case before, but is established, starting from 2021, taking into account the median salary [12, 13]. More details on the salaries under consideration are presented in Figure 7.

Thus, for the Veterinary Service as a whole, the arithmetic mean salary was 42 thousand rubles, but such salary or higher was paid to the veterinary specialists only in 25 Subjects of the country. The veterinary specialist salary in half of the Russian Federation regions exceeded 37 thousand rubles and was below this amount in the other half of the regions. The most frequent salary paid to this category of the State Veterinary Service staff falls within the range of 35 to 40 thousand rubles and amounts to about 36 thousand rubles. Thus, the calculations performed allowed for the assessment of the state veterinary institutions' staff remuneration from different viewpoints.

The data presented in the paper show that the salary level for most of the veterinary specialists of the State Veterinary Service of the Russian Federation is low; besides, there is a large difference in the income of the specialists working in different regions of the country. In our view, this is not quite fair. It may be appropriate, when establishing the veterinary specialist salary, to follow the principle applied to the establishment of the medical worker salary, since these professions are similar in many aspects, so such approach would be rather reasonable, in our opinion. In accordance with the "May Decrees" of the Russian Federation President, the medical practitioner (doctor) average salary shall make up 200% of the average for the relevant region [14]. There is also the Country Doctor programme in place, which envisages the payment of up to 2 million rubles for the medical workers that become employed in the countryside under certain conditions [15]. In addition, pursuant to Decree of the Government of the Russian Federation No. 847 of 1 June 2021, a pilot project aimed at the further improvement of the medical worker remuneration system was launched in 7 regions of the country in 2022 [16].

Thus, new approaches are constantly being developed for the regulation of medical worker remuneration. We would like to see the same in the veterinary sphere. It is fair to point out that a new section regarding the specific features of the remuneration system for the staff members of the state veterinary institutions was first included in the Uniform recommendations for the establishment of remuneration systems for employees of state and municipal institutions at the federal, regional and local levels for 2020 (hereinafter referred to as the Uniform recommendations) [17]. However, the Uniform recommendations for 2020 and those for 2021 do not specify the indicative lower limit for the veterinary specialist salary, but only state that the salary in each of these periods should not be lower than that in the previous year [18]. An addition providing for the progressive salary increase to achieve a level that will not be lower than the average monthly salary for the relevant Russian Federation Subject was made only to the Uniform recommendations for 2022 [19]. Similar recommendations are laid down in Ordinance of the Government of the Russian Federation No. 3789-r of 7 December 2022 [20]. It is, therefore, hoped that the veterinary specialist salary situation in the Russian Federation Subjects will be more optimistic starting from 2023.

In many regions of the Russian Federation, the relatively low remuneration of the state veterinary institutions' staff for the analyzed year (2021) was partially compensated by various social support measures for the veterinary specialists. In particular, based on the data from the veterinary executive authorities of the Russian Federation Subjects for 2021, 24 regions developed a programme for the improvement of the staffing of the Veterinary Service of the relevant Subject (including the attraction of young professionals); 38 Subjects have a procedure in place for the provision of a dwelling or payments to buy and rent it; 57 regions are implementing a procedure for the target admission to the higher educational institutions for the specialty "veterinary medicine". Unfortunately, only 40% of young specialists became employed in the State Veterinary Service Institutions over the past 10 years.

The comparative analysis of salaries of medical workers and veterinary specialists across the federal districts revealed the following regularity: the veterinary specialist salary level tends to be higher in the districts with the higher medical worker salary level [21].

Each region deals with the veterinary specialist remuneration issue on its own, depending on the local budget available; there is, therefore, a need for the federal material support programme for the veterinary specialists (especially, early-career ones). A low salary makes the profession of a veterinarian less attractive, and this results in incomplete staffing with veterinary specialists in the regions: such vacancies are fully filled in only 7 out of 85 Subjects. That said, the animal and human disease freedom of the country directly depends on the work of the veterinary specialists.

CONCLUSION

The analysis of the veterinary specialist salary data provided by the veterinary executive authorities of the Russian Federation Subjects allowed for the assessment of remuneration of the state veterinary institutions' staff for the country as a whole, as well as for the federal districts and individual Subjects.

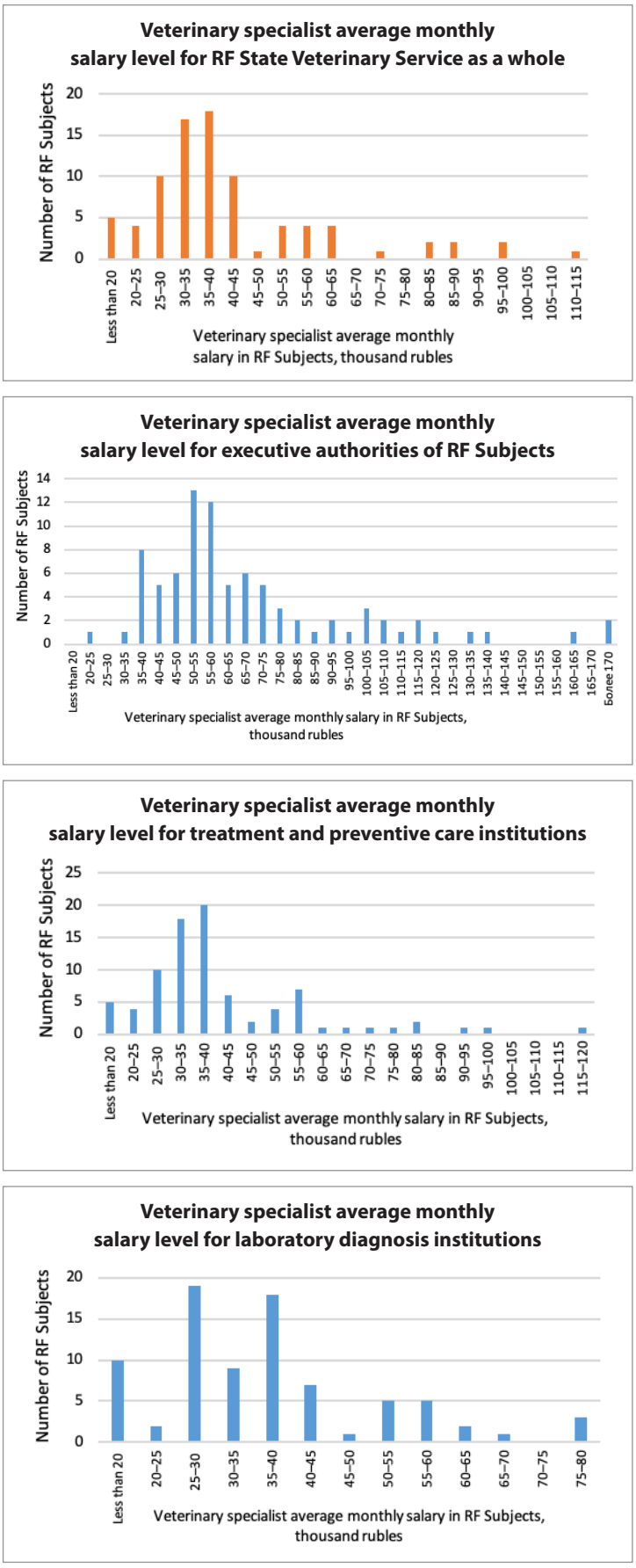
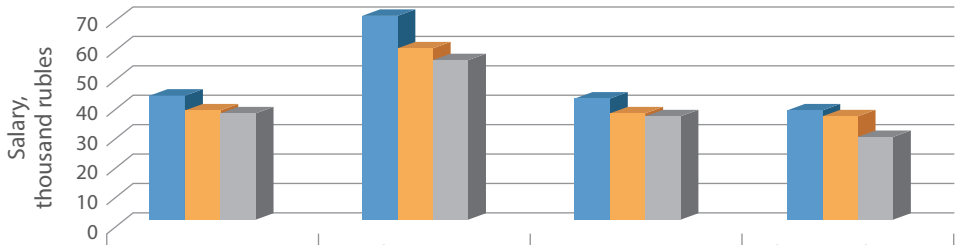


Fig. 6. Distribution of the Russian Federation Subjects by veterinary specialist salary at different organizational levels within the Veterinary Service



	Veterinary Service as a whole	RF Subject executive authorities	Treatment and preventive care institutions	Laboratory diagnosis institutions
Arithmetic mean	42	69	41	37
Median	37	58	36	35
Modal	36	54	35	28

Fig. 7. Comparative data on arithmetic mean, modal and median salaries for veterinary specialists of the State Veterinary Service of the Russian Federation

The funding allocated for salaries was mainly sourced from the budgets of the Russian Federation Subjects, which accounted for 61% of the total funding intended for the payment of staff salaries.

The veterinary specialist average monthly salary varies significantly across the Russian Federation Subjects (7-fold). In 69 out of 85 regions of the Russian Federation, the veterinary specialist salary for the Veterinary Service as a whole is lower than the average monthly salary for the economy of these Subjects.

It was found that the most frequent salary paid to this category of staff falls within the range of 35 to 40 thousand rubles and amounts to about 36 thousand rubles. The veterinary specialist remuneration for the Veterinary Service as a whole exceeds 37 thousand rubles a month in half of the Russian Federation Subjects and is below this amount in the other half.

Low salaries of the veterinary specialists of the State Veterinary Service are indicative of insufficient funding in this sphere. In our opinion, the fair regulation of remuneration requires taking additional measures enshrined in the federal programmes. Such programmes can, for example, envisage material rewards for the Veterinary Services of the Subjects for the maintenance of highly dangerous animal disease freedom. However, this proposal requires a thorough consideration due to the possibility of illusory animal disease freedom in the territories under their responsibility. A disease free status granted to the relevant region with respect to a particular animal infectious disease within the framework of regionalization of the Russian Federation may become a ground for incentive payments to the veterinary specialists of the regional Veterinary Services [22, 23]. The recognition of such status enhances the significance of the region as regards the attraction of investment to the economy of the Subject, in particular to the livestock sector and agriculture as a whole.

Such annual incentive payments can also be legislatively established at the regional level, i.e. additional payments can be made from the Subject budget to the veterinary specialists of the municipal districts that have been free from highly dangerous animal diseases during the year. In our view, such approach will encourage the veterinarians of each Subject / municipal district to make the utmost effort to maintain animal disease freedom in their region. All this requires deep knowledge, well-honed skills and continuous professional improvement. That is why the work of the veterinary specialists in our country should be decently paid for.

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