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SEPTEMBER | СЕНТЯБРЬ VOL. 13 No. 3 2024

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World RABIES Day

SEPTEMBER 28

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AIMS AND SCOPE

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

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

ЦЕЛИ И ОБЛАСТЬ (ТЕМАТИЧЕСКИЙ ОХВАТ)

Миссией издания является представление информации об основных направлениях развития российской и мировой ветеринарной науки и практики и привлечение внимания научной общественности к актуальным проблемам и инновационным разработкам в области ветеринарии.

Журнал ориентирован на ученых, занимающихся фундаментальными и прикладными исследованиями в области общей и ветеринарной вирусологии, эпизоотологии, иммунологии, микологии, микотоксикологии, бактериологии, практикующих ветеринарных врачей и врачей ветеринарных лабораторий и государственных ветеринарных служб, преподавателей вузов ветеринарной, биологической, медицинской направленностей, аспирантов и студентов вузов и колледжей.

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From the Editor-in-Chief of "Veterinary Science Today"

Dear readers and colleagues!

The editorial board of "Veterinary Science Today" presents the next issue of the journal dedicated to the World Rabies Day, which is celebrated annually on September 28.

Rabies is a viral disease that affects the central nervous system of mammals (dogs, cats, foxes, etc.), including humans. According to the World Organization for Animal Health, the rabies virus is present in the saliva and brain of infected animals and is transmitted through a bite. The main reservoir of the pathogen is wild carnivores, and in some regions, bats. According to the World Health Organization, rabies is one of the five zoonotic diseases of great social importance, poses a mortal threat to humans and animals, and kills about 59,000 people annually. The human mortality rate is 100%. Thanks to modern scientific advances, veterinary services in many European countries succeeded to eradicate this disease. However, in most countries of the world, including Russia, the rabies situation remains tense.

The global initiative of the World Health Organization, the Food and Agriculture Organization of the United Nations, and the World Organization for Animal Health to eliminate dog-mediated human rabies by 2030 has received worldwide support. In our country, a number of legal measures have been taken concerning rabies prevention, epidemiological monitoring, research expansion and awareness-raising campaigns in the media.

We encourage everyone to contribute to the solution of the rabies problem.

Sincerely,
Editor-in-Chief of the Journal,
Doctor of Biological Sciences,
Professor Konstantin N. Gruzdev

The editorial board of "Veterinary Science Today" congratulates Dr. Emmanuelle Soubeyran on her appointment as Director General of the World Organization for Animal Health.

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Alternative treatment methods for bovine mastitis: prospects and limitations (review)

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ABSTRACT

Mastitis remains the most common problem of dairy industry despite the preventive measures and treatment schemes being developed. Antibacterial drugs remain first line agents for therapy of the mammary gland inflammatory diseases in animals. Taking into account the risks associated with antibiotic therapy, such as decreased drug effectiveness due to occurrence of bacterial resistant strains, food safety issues, environmental impact and restrictions on the use of antibacterial drugs in veterinary medicine, an increasing number of scientific studies are addressing new therapeutic agents that can serve as an alternative to conventional therapy. The aim of this review is to give an idea of currently available literature data on alternative methods for the prevention and treatment of mastitis in cattle that are not associated with antibiotics. In general, a significant number of *in vitro* studies aimed at finding new effective and safe drugs are yielding promising results. This review describes the following alternative remedies: probiotics, bacteriocins, bacteriophages, phage enzymes (endolysins), nanoparticles, plant extracts, essential oils and immunobiological agents (vaccines). Understanding the mechanisms of their action will allow recommending the best treatment option for mastitis in each specific case. These treatment methods can potentially reduce use of antibiotics and increase animal productivity, however more *in vivo* studies are needed to prove the effectiveness of antibiotics used directly in the conditions of farm settings.

Keywords: review, mastitis, alternative treatments, probiotics, bacteriocins, bacteriophages, endolysins, nanoparticles, herbal extracts, essential oils, immunobiological prevention

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Альтернативные методы лечения мастита крупного рогатого скота: перспективы и ограничения (обзор)

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РЕЗЮМЕ

Мастит продолжает оставаться наиболее распространенной проблемой молочного животноводства, несмотря на разрабатываемые профилактические меры и схемы лечения. Антибактериальные препараты являются основным средством терапии при воспалительных заболеваниях молочной железы у животных. Принимая во внимание связанные с антибиотикотерапией риски, такие как снижение эффективности действия препаратов из-за появления резистентных штаммов бактерий, проблема безопасности пищевых продуктов, воздействие на окружающую среду и введение ограничений на применение антибактериальных препаратов в ветеринарной медицине, все большее количество научных исследований обращается к новым терапевтическим средствам, которые могут стать заменой традиционной терапии. Цель настоящего обзора – дать представление о доступных в настоящее время литературных данных по исследованию альтернативных методов профилактики и лечения мастита крупного рогатого скота, не связанных с антибиотиками. В целом существует огромное количество исследований *in vitro*, направленных на исследование новых эффективных и безопасных средств, которые дают многообещающие результаты. В данном обзоре описаны такие средства, как пробиотики, бактериоцины, бактериофаги, фаговые ферменты (эндолизины), наночастицы, растительные экстракты, эфирные масла и иммунобиологические средства (вакцины). Рассмотрены механизмы их действия, понимание которых позволит рекомендовать наилучший вариант лечения мастита в каждом конкретном случае. Данные методы терапии потенциально могут сократить использование антибиотиков и повысить продуктивность животных, однако требуется больше исследований *in vivo,* чтобы доказать эффективность их применения непосредственно в условиях сельскохозяйственных организаций.

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Ключевые слова: обзор, мастит, альтернативные методы лечения, пробиотики, бактериоцины, бактериофаги, эндолизины, наночастицы, экстракты трав, эфирные масла, иммунобиологическая профилактика

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INTRODUCTION

Bovine mastitis, or mammary gland inflammation in livestock, is the most common disease of dairy cows that incurs losses in agriculture. It was established that about 150 different bacterial species/subspecies are capable of causing this disease in cattle. However, more than 95% of mastitis cases are associated with only 10 groups of microorganisms, including both opportunistic and pathogenic ones, depending on their reservoir and transmission method [1]. Such bacteria include *Staphylococcus aureus*, *Mycoplasma* spp., *Streptococcus uberis*, *Streptococcus dysgalactiae*, coliforms and other gram-negative bacteria such as *Escherichia coli* and *Klebsiella pneumoniae*. Other agents, such as *Arcanobacterium pyogenes*, various streptococci (*Streptococcus parauberis*, *Streptococcus agalactiae*, *Streptococcus zooepidemicus*), *Corynebacterium bovis* and *Mycobacterium bovis*, may be involved in the inflammatory process to a lesser extent [2].

Antibiotics are considered the frontline drugs in the treatment of this inflammatory process. However, the issue of antimicrobial residues in animal products and the continuous growth of antimicrobial resistance, together with the possible spill-over of antibiotic-resistant bacteria from animals to humans, results in restrictions on the use of these products in veterinary medicine [3]. The development and introduction of new classes of antibiotics may seem the most obvious strategy, but since 1987 not a single class of antibiotics has been discovered and only derivatives of existing antibacterial drugs have been used [4, 5]. The discovery of several classes of antibiotics in a short period of time has led to their overuse, as well as to a rapid increase in the number of microorganisms with antibiotic resistance genes. In the 1990s such companies as Pfizer, AstraZeneca and GlaxoSmithKline performed studies on potentially new antibacterial targets for antibiotic development, but no suitable candidate was found [6]. Studies of pharmaceutical companies are aimed at modifying existing classes of antibiotics, rather than developing potentially new ones [7]. In this regard, there is currently a need to develop alternative means for the prevention and control of bovine mastitis.

The aim of this review is to give an idea of the latest discoveries related to alternative means, including probiotics, bacteriocins, bacteriophages (phages) and phage enzymes, nanoparticles, herbal extracts, essential oils and immunobiologicals (vaccines) for prevention and treatment of bovine mastitis. Systematized and generalized information and literature sources within the review scope [8–42] are presented in Table 1 in the Additional Files section at: https://doi.org/10.29326/2304-196X-2024-13-3-203-213.

PROBIOTICS

According to modern concepts, mastitis is developed due to imbalance of the mammary gland microbiota, therefore probiotics are viewed as alternative preventive and therapeutic means. Intramammary inoculation of probiotics (lactic acid-producing bacteria) leads to their colonization in the udder [43]. The mechanisms of probiotic activity against pathogenic microorganisms are as follows: adhesion to epithelial cells, aggregation and coagulation, biofilm formation, colonization, production of biosurfactants and/or antagonistic metabolites (organic acids, hydrogen peroxide, bacteriocins), competition for nutrients and/or enzyme production [11]. Probiotic bacteria can be used to control inflammatory processes, especially in the dry season, due to antagonistic activity against mastitis etiological agents and through immunomodulation, namely by influencing the development, differentiation and effector functions of a wide range of subpopulations of immune cells, as well as epithelial cells [11, 44, 45, 46]. In addition to intramammary use, probiotics can also be used as disinfectants, nipple treatments before and after milking [9, 47].

Modern studies are devoted to probiotics used for prevention and treatment of mastitis that contain *Lactococcus lactis*, *Lactobacillus perolens*, *Lactobacillus paracasei*, *Lactobacillus plantarum*, *Lactobacillus casei*, *Lactobacillus rhamnosus*, *Schleiferilactobacillus perolens*, *Bifidobacterium breve*, *Bacillus subtilis*.

Many scientists note the potential of probiotics against the most common mastitis pathogens: *S. aureus*, *Staphylococcus epidermidis*, *Staphylococcus chromogenes*, *Staphylococcus intermedius*, *S. agalactiae*, *S. dysgalactiae* and *E. coli* [8], however, these studies were mainly conducted *in vitro*. The mechanism of action of many lactic acid bacteria as probiotics is the inhibition of aggregation of bacterial pathogens to mammary epithelial cells (MAC-T) [19] and the secretion of antimicrobial substances (bacteriocins) [9].

Researchers from Argentina studied 12 species of lactic acid bacteria. Two of them, *L. lactis* subsp. *lactis* CRL 1655 and *L. perolens* CRL 1724, are capable of adhesion to mammary epithelial cells, inhibition and coagulation of 15 *S. aureus*

strains. For mastitis prevention Pellegrino M. et al. recommend intramammary inoculation of these probiotics to cows at dry-off period to activate the immune response by triggering the production of specific antibodies [11].

Another feature of some lactobacilli is the production of their own biofilms. *L. rhamnosus* ATCC 7469 and *L. plantarum* 2/37 strains have the ability to disrupt pathogenic staphylococcal biofilms and replace them with biofilms of their own [12].

The Chinese researchers [15] note the effectiveness of *L. rhamnosus* GR-1-based probiotic for coliform mastitis. This strain of lactic acid bacteria blocks the production of reactive oxygen species and mediates the activation of mitophagy, thereby inhibiting *E. coli*-induced assembly of NLRP3 inflammasome of the family of NLR receptors (NOD-like receptors) that causes apoptosis of mammary epithelial cells. Thus, the use of probiotics promotes the activation of mitophagy and the preservation of mitochondrial cell function.

Qiu M. et al. studied the mechanism of action of *Enterococcus mundtii* H81 in mammary inflammation and for that they used mice models with *S. aureus*-induced mastitis. *E. mundtii* H81 was found to have ability to inhibit *S. aureus* growth. The H81 strain protects the integrity of the mammary epithelial barrier. The results demonstrated that *E. mundtii* H81 reduces pathological damage to mammary tissue by reducing the secretion of proinflammatory cytokines and inhibiting the activation of the signaling pathway of the nuclear transcription factor NF-kB. Consequently, *E. mundtii* H81 may have potential as a promising candidate for the treatment of *S. aureus*-induced mastitis [17].

A number of experiments are aimed at studying the probiotic potential of lactic acid bacteria to better understand how these properties can be used for *in vivo* control of bovine mastitis pathogens.

Intramammary administration of the *L. lactis* probiotic strain proved to be as effective as the conventional antibiotic for the treatment of various forms of mastitis. In this case, the lactococci were completely eliminated from the treated gland after a few days. Many researchers assume that reconvalescence occurs due to induced local inflammation, intensive involvement of leukocytes and stimulation of mammary protection [9, 10, 13].

Catozzi C. et al. [14] investigated intramammary administration of *L. rhamnosus* in buffaloes with a subclinical form of mastitis and observed proinflammatory activity and modification of the milk microbiota. Treatment with *L. rhamnosus* elicited a strong chemotactic response, as determined by a significant increase of leukocytes in milk. Concerning the analysis of the microbiota, the treatment induced the modification in relative abundance of some genera such as *Pseudomonas* spp. and 5-7N15. Initially, there was an increase in the number of somatic cells in milk, but after 6 days the number of somatic cells decreased significantly. A similar response was observed with intramammary infusion of *B. breve* [16]. In this regard, further studies are needed to assess the potential use of GRAS (Generally Recognized as Safe) bacteria as a maintenance treatment against mastitis.

Oral administration of probiotic strains is an alternative for the prevention and treatment of mastitis. As shown by M. Urakawa et al., the introduction of a *B-subtilis* C-3102-based feed additive into the diet leads to a significant reduction in the incidence of mastitis, as well as maintaining the mean value of somatic cells in milk at a level significantly lower than in the control group. Besides, the experimental group had lower levels of cortisol and reactive compounds of thiobarbituric acid, consequently, the cows did not experience oxidative stress. The flow cytometry showed an increase in the proportion of CD4+ T-cells and CD11c + CD172a^{high} dendritic cells in the blood. Dendritic cells are antigen-presenting cells specializing in the absorption and processing of antigen, which play an important role in innate and adaptive immune responses. The data show that *B. subtilis* C-3102 can be used for prevention of bovine mastitis [18].

In general, the studies described above show that probiotic strains have great potential for the development of effective means for the treatment and prevention of mastitis, but their effectiveness in the treatment of the clinical disease form has yet to be determined.

BACTERIOCINS

Bacteriocins are bacterial peptides synthesized on ribosomes that show antimicrobial activity against other bacteria, including antibiotic-resistant strains [44]. Some bacteriocins (e.g., nisin produced by *L. lactis*) are already used for food preservation due to their antimicrobial effectiveness and at the same time a high degree of safety for consumers [3]. In practice, either purified bacteriocins administered directly in their purest form, or viable bacteria producing bacteriocins (mainly lactic acid) are applied [13]. The sensitivity of bacteria to bacteriocins is associated with their interaction with the bacterial cell surface and cell membrane. Cell permeabilization and pore formation represent the main mechanism by which bacteriocins attack target bacteria. Since the surface charge of the plasmolemma and the fluidity of the membrane are two bacterial properties used as targets for bacteriocins, changing these properties makes bacteriocins ineffective, which leads to the development of resistance to bacteriocins [48]. However, this resistance can be overcome by using bacteriocin combinations [49] with each other or with other antimicrobial compounds [50]. In addition, the effectiveness of bacteriocins can be increased through bioengineering. As bacteriocins, unlike antibiotics, are ribosomally synthesized peptides, their amino acid residues can be altered, thus inducing their antimicrobial effect. Bacteriocins are generally divided into 3 classes (Table 2) [48].

A drug containing bacteriocin produced by *Streptococcus equinus* HC5 has been developed for the treatment of bovine mastitis. Bovicin HC5 has some similarities with nisin as regards its mechanism of action, since it is able to bind to lipid II in the cytoplasmic membrane. Brazilian researchers studied the activity of bovicin HC5 against pure or mixed cultures of staphylococci, streptococci and escherichia strains isolated from cows diagnosed with mastitis in various dairy herds, and confirmed its ability to inhibit the growth of more than 80% of the tested streptococci and staphylococci strains, but noted that no antimicrobial effect against *E. coli* strains was observed [20].

Scientists from Thailand studied the antimicrobial potential of the non-ribosomal peptide Pm11, which is produced from pleurocidin, belonging to the family of cationic α-helical peptides found in *Pleuronectes americanus*. In this study, the Pm11 peptide was found to be active against *E. coli* SCM1249, *S. aureus* CM967, *S. agalactiae* SCM1084 and *S. uberis* SCM1310 strains. However, no antimicrobial

Table 2
Classification of bacteriocins

Class		Features	Producers	Example	Mechanism of action
I	Ia	Lantibiotics (< 5 kDa peptides containing lanthionine and β-methyl lanthionine)	*L. lactis*	Nisin	Cell permeabilization and pore formation, lipid II receptor, action against gram-positive bacteria
	Ib	Carbocyclic lanthibiotics containing labyrinthine and labionine	*Actinomadura namibiensis*	Labyrinthopeptin A1	Herpes simplex virus (HSV) and human immunodeficiency virus (HIV)
	Ic	Sactibiotics (sulphur-to-α-carbon-containing antibiotics)	*Bacillus thuringiensis*	Thuricin CD	Gram-positive bacteria
II	IIa	Small heat-stable peptides, synthesized in a form of precursor which is processed after two glycine residues	*Pediococcus pentosaceus*, *Pediococcus acidilactici*, *Lactobacillus sakei*	Pediocin PA-1, sacacins A and R, leukocin A	Cell permeabilization and pore formation, mannose permease receptor. Active against gram-positive and gram-negative bacteria, active against listeria
	IIb	Two-component systems: two different peptides required to form an active poration complex	*L. lactis* subsp. *cremoris*, *L. plantarum*	Lactococcins G, plantaricin EF and plantaricin JK	Cell permeabilization and pore formation, UppP receptor (undecaprenyl pyrophosphate phosphatase), action against gram-positive bacteria
	IIc	Circular bacteriocins	*Lactobacillus gasseri*, *E. faecalis*, *Lactococcus garvieae*	Gassericin A, enterocin AS-48, garvicin ML	Cell permeabilization and pore formation, ABC receptor transporter, action against gram-positive bacteria
	IId	Unmodified, linear, leaderless, nonpediocin-like bacteriocins	*Lactobacillus salivarius*, *L. lactis* subsp. *lactis*	Bactofencin A, LsbB	Cell permeabilization and pore formation, metallopeptidase receptor, action against gram-positive bacteria
III		Large molecules sensitive to heat	*Lactobacillus crispatus*, *Lactobacillus helveticus*, *E. faecalis*	Helveticin M, helveticin J and enterolysin A	Cell permeabilization and pore formation, action against gram-positive and gram-negative bacteria

activity was observed against the *Klebsiella* spp. SCM1282 strain due to the presence of an extracellular polysaccharide capsule in these microorganisms. When the peptide interacts with the bacterial capsule, its structural changes occur, causing sequestration and preventing the peptide from reaching its pathogen membrane target [21].

Garvicin is class II bacteriocin produced by *L. garvieae* strains [24]. Norwegian researchers identified the inhibitory ability of garvicin KS against *Acinetobacter baumannii*. When used in combination with nisin, garvicin also inhibits *S. aureus* growth [25].

In another study, Brazilian scientists studied the antagonistic activity of aureocin 4181, a staphylococcin produced by *S. aureus*. This bacteriocin has proven effective against a wide range of gram-positive bacteria, including other strains of staphylococci and streptococci [26]. The bactericidal mode of action of aureocin is associated with the destruction of cell membranes of mastitis pathogens [51].

Bactofencin A was isolated from gram-positive *L. salivarius* [52] and demonstrated inhibitory activity against *S. aureus* and *Listeria monocytogenes* by acting on the bacterial cell wall [22]. Nisin A, a lantibiotic produced by *L. lactis*, exhibits broad-spectrum activity against gram-positive bacteria. Its mode of action is based on the destruction of the bacterial cell wall by pore formation and inhibition of the biosynthesis of important cell wall precursors. *Lactobacillus reuteri* generates an active aldehyde known as reuterin in the presence of glycerin. This compound was found to be effective against a wide range of gram-positive and gram-negative bacteria because it causes oxidative stress in cells. Several studies were aimed at evaluating the potential of reuterin as a food preservative [53] and disinfectant [54]. Canadian scientists studied the antibacterial effect of bactofencin A, nisin and reuterin bacteriocins both individually and in combination, using them as a means to treat udder nipples before and after milking. The conducted research showed that the use of bactofencin A did not reduce the amount of staphylococci and streptococci on the surface of udder nipples; nisin and reuterin, on the contrary, reduced bacterial contamination. When these bacteriocins were used in combination, the most pronounced antibacterial effect similar to the biocidal action of nisin and iodine was observed. Thus, the combined use of several bacteriocins has many advantages [23]. Xu X. et al. demonstrated that lower concentrations of antimicrobials with synergistic effects are needed to inhibit bacterial growth [55]. Consequently, it reduces treatment costs and risks of adverse effects caused by drug toxic effect [23]. In addition, bacteriocins can be used in combination with antibacterial drugs. For example, nisin A increases the activity of cephazolinum, thereby reducing the dose of the antibiotic in the mastitis treatment. This combination is effective against *S. aureus*, *S. intermedius*, *S. agalactiae*, *S. dysgalactiae*, *Enterococcus faecalis* and *E. coli* [24].

The rapid discovery of new bacteriocins, their development and combination with other bactericidal agents inevitably leads to increased resistance to these drugs. The potential hepatotoxicity of these bacterial peptides should also be taken into account [48]. In general, various approaches are to be considered to solve the issue of resistance and reduce the toxicity of bacteriocins, which have great potential as bioconservants and therapeutic agents.

BACTERIOPHAGES

Bacteriophages (phages) specifically infect bacteria, resulting in either lysis of the bacterial agent (lytic or virulent phages), or in lysogeny – the integration of the bacteriophage's genetic material into the bacterial chromosome

of the host (moderate or symbiotic phages) [56]. Bacteriophages, due to the specificity of their action, cause minimal disruption of the normal microbiome of animals, thereby not causing dysbiosis [57]. Such selectivity of bacterial targets by phages is achieved by recognizing specific receptor proteins located in the bacterial cell wall, on which the phage is adsorbed using specialized fibrils, after which bacteriophages penetrate and release their genetic material in the bacterial cell [58]. As a rule, phages of most *S. aureus* strains interact in the cell wall with teichoic acid, which differs from other acids inherent in coagulase-negative staphylococci [59]. For studies aimed at searching bacteriophages acting against one of the main pathogens of mastitis – *S. aureus*, the following main domains located in endolysin sequences are used: cysteine, histidine-dependent amidohydrolase/peptidase (CHAP), amidase 2 (N-acetylmuramoyl-L-alanine amidase) and SH3b for recognition of the cell wall of the pathogenic agent [60].

Following successful adsorption and penetration into the cell, lytic phages capture the mechanism of bacterial DNA replication to synthesize their own genetic material and structural proteins during the latent period. The duration of the period required to start synthesis varies for bacteriophages acting against bovine mastitis pathogens and can be 5 (*E. faecalis*), 10 (*S. aureus*), 20 (*Pseudomonas aeruginosa*) or 30 minutes (*S. agalactiae*) [61, 62, 63, 64]. Subsequently, after viral synthesis, numerous phage particles are assembled and eventually released by the lysis of the host through a combined activity of the endolysin and holin enzymes that degrade the bacteria cell wall [57]. In case of bovine mastitis, the number of phage particles synthesized and released per bacterial cell varies from 20 to 100 PFU/cell (plaque-forming units per 1 cell) for approximately 175 minutes [61, 62, 63, 64]. The ability of lytic phages to eventually lyse bacteria and replicate after infection ensures the destruction of bacterial pathogens, as well as a constant increase in the concentration of infectious phages (auto-dosing) at the site of infection [65]. In addition, the short replication time demonstrated by phages makes it possible to shorten the drug development timeline, providing the opportunity for rapid individual treatment aimed at specific bacterial strains [57].

Many studies have noted a significant decrease in bacterial load during exposure of phages against pathogenic agents that cause mastitis [27, 28, 29, 30, 31]. However, the resistance was detected within two hours after phage treatment, as evidenced by the resumption of bacterial growth after lysis, which may adversely affect therapeutic efficacy [28]. In order to limit the development of resistance and lysogeny, increase the specificity of the target bacteria and raise lysis efficiency, it is possible to optimize the composition of the phage cocktail [66, 67].

For instance, I. Titze and V. Krömker investigated the efficacy of bacteriophage mixture with *L. plantarum* on *S. aureus* strains isolated from the milk of cows with mammary gland inflammation. The phage cocktail, as well as its combination with lactic acid bacteria, demonstrated high antimicrobial activity against *S. aureus* during a 24-hour incubation period at 37 °C. Statistical calculations showed that only a bacteriophage mixture had a significant effect on the growth rate of *S. aureus* [32].

The Chinese researchers assessed the antibacterial activity of bacteriophage mixtures experimentally. For this purpose, eight lactating Holstein cows were selected and randomly divided into four groups, two animals per each group. Three groups of cows were intramammarly inoculated with 60 CFU *E. coli* ECD2 suspended in 1 mL of pyrogen-free phosphate buffer saline solution (PBS). Phage cocktails containing SYGD1, SYGE1, and SYGMH1 were prepared by mixing the three phages at a 1:1:1 ratio with a primary concentration of about 10^{10} PFU/mL. The mixture was 100-fold diluted with PBS. One group was intramammarly inoculated with 5 mL ceftiofur sodium (600 mg/mL), the second group was intramammarly inoculated with 5 mL phage cocktails (1×10^8 PFU/mL), the third group was intramammarly inoculated with 5 mL PBS only. All products were administered once a day for three consecutive days. The fourth group, as a control group, was neither inoculated nor treated. All three bacteriophages showed promising results as antimicrobial agents, especially when used in a cocktail, such therapy can reduce the number of bacteria, somatic cells and inflammatory factors, alleviate the symptoms of mastitis in cattle and achieve the same effect as with antibiotic treatment [33].

Pathogens causing mastitis are capable of forming biofilms, which limits the access of antibiotics to bacteria [68, 69, 70]. However, phages can prevent biofilm formation or penetrate bacterial pathogens *in vitro* and *in vivo*, which indicates the possibility of their use as an independent treatment or in combination with antibiotics to increase therapeutic effect [28, 69]. In a study by Iranian scientists, bacteriophage M8 showed noticeable lytic activity against all tested types of *S. aureus* (multi-resistant, methicillin-resistant and biofilm-forming strains). This bacteriophage, alone or in combination with other phages and antibiotics, has the potential of being a therapeutic option for intractable inflammatory mammary diseases caused by *S. aureus* [34].

The results of many *in vitro* and *in vivo* studies show that phage therapy is a promising alternative to antibiotics for the treatment of mastitis in cows, and in combination with antimicrobials will reduce the dose of the latter or shorten the period of treatment [71]. However, the efficacy of phage therapy is limited due to their strict specificity against certain combinations of mastitis pathogen strains and the need to use several phages to control a variety of bacterial pathogens. Phage therapy is most effective when the target pathogen is readily available and present in large quantities [72].

PHAGE ENZYMES

One of the ways to handle challenges of phage therapy may be the use of purified products of phage genes, such as lysines. Endolysins (amidase, endopeptidase, glucosidase and transglycosylase), commonly known as enzybiotics, are mureolytic enzymes that are synthesized at the end of the phage lytic life cycle [73]. They impact peptidoglycan bonds and lyse bacteria from the inside, promoting the release of new phages. Endolysins have a wider antibacterial spectrum compared to phages. In addition, they can also lyse bacteria when used exogenously. Endolysins are specific, highly active and carry a lower risk of developing resistance [74].

The well-studied and most active lysines include streptococcal-specific lysine PlyC obtained from bacteriophage C1. Although almost all gram-positive endolysins described to date are encoded by a single gene, the endolysin PlyC of the C1 phage of group A streptococcus

is the only example of a multimeric lysine consisting of two different gene products: PlyCA and PlyCB. One PlyCA subunit with enzymatic activity and eight PlyCB subunits that make up the cell wall binding domain form a complete PlyC complex, which is an endolysin with the highest activity, just one nanogram is enough to destroy 10^7 CFU of various streptococcal species in a few seconds [3, 35, 75].

Schmelcher M. et al. studied the possibility of using endolysins of *λ*SA2 and B30 streptococcal phages as antimastitis agents in 2015. Lysine *λ*SA2 showed high activity in cow's milk against *S. dysgalactiae*, *S. agalactiae* and *S. uberis*, whereas lysine B30 was less effective. Both enzymes significantly reduced the concentration of all three types of streptococci in the mouse mastitis model (with the exception of B30 relative to *S. dysgalactiae*). It is worth noting that the synergistic effect found for the two enzymes *in vitro* was not observed in the mouse model. In general, the results obtained demonstrate the potential of endolysins for the treatment of streptococcus-induce bovine mastitis [36].

A study by the Chinese scientists has shown that LysKΔamidase endolysin is able to inhibit 71 methicillin-sensitive and 66 methicillin-resistant staphylococcus strains isolated from the milk of cows with mastitis. The wide antistaphylococcal *in vitro* activity of this enzyme, including that against multidrug-resistant staphylococci and biofilm-producing staphylococci, indicates that LysKΔamidase can become a means of therapy for intractable inflammatory diseases of mammary glands [37].

However, the number of clinical studies on the use of endolysins for the treatment of bovine mastitis is limited. In one of such experiments J. Fan et al. intramammarly administered 20 mg of endolysin Trx-SA1 to cows at the initial stage of clinical mastitis once a day for 3 days. In 60% of cases, milk samples demonstrated decrease in *S. aureus* total count and the number of somatic cells [38].

Despite the promising prospects for the use of endolysins as a therapeutic means for mastitis, their use requires further study as there are certain limitations. For example, repeated administration of lysing proteins leads to the formation of immunoglobulins against the inoculated phage enzymes, which limit the antimicrobial activity of the latter [44]. In addition, most endolysins are not active against gram-negative bacteria, since the outer membrane protects the underlying carbohydrates and peptidoglycan from direct contact with lysines. Nevertheless, one of the main advantages of using bacteriophages and phage endolysins is their ability to eliminate antibiotic-resistant pathogens against which conventional therapeutic methods are ineffective [38].

NANOPARTICLES, PLANT EXTRACTS AND ESSENTIAL OILS

In addition to the above-mentioned means of mastitis therapy and prevention, relatively new control strategies include the use of nanoparticles, herbal extracts and essential oils.

Nanoparticles have broad-spectrum antimicrobial potential and do not affect the development of resistance in bacteria. The antimicrobial effect of metal nanoparticles is explained by: 1) release of the resulting active oxygen; 2) peroxidation of bacterial proteins and lipids; 3) penetration of carbohydrates into bacterial cells; 4) degradation of microbial DNA; 5) damage to the cell membrane and, as a result, an increase in its permeability [76, 77]. After exposure of nanoparticles on bacteria, a decrease in lactate dehydrogenase activity and adenosine triphosphate levels was observed, which indicates ineffective energy regulation in mastitis pathogens. There is also downregulated gene expression in pathogens, including genes encoding glutathione (GSH), glutathione S-transferase (GST), superoxide dismutase (SOD) and catalase (CAT), which induces bacterial death [77]. The results obtained during the pilot studies showed that copper nanoparticles inhibit *S. aureus* growth and exhibit minimal toxicity to fibroblast cell lines at a concentration of 6.25 μg/mL. Intramuscular administration of copper nanoparticles to rats with staphylococcus-induced mastitis turned out to be more effective than gentamicin injections; these conclusions were made based on clinical signs, results of total bacterial load and the study of histological specimens [39].

However, as the use of nanoparticles in mastitis therapy has not yet become widespread as an alternative to the classical approach using antibiotics, many researchers prefer combination therapy including nanoparticles and antimicrobials. It is already known that intramammary administration of the drug with nanosilver and ceftiofur has therapeutic efficacy up to 93.33% of cases. This combination can also be used for preventive purposes, for example before calving [78].

The use of plant extracts and essential oils in the treatment of mastitis is a fairly promising area of research, since, compared with antibiotics, these drugs have a natural composition, they do not have severe side effects [79], and plant components do not participate in bacterial resistance in bacteria after prolonged exposure [80]. This method of mastitis treatment in food-producing animals has been known for a long time, extracts of plants such as *Taraxacum mongolicum*, *Lonicera japonica*, *Viola patrinii*, *Folium isatidis*, *Angelica dahurica*, *Coptis chinensis*, *Phellodendron amurense*, *Rheum officinale*, *Scutellaria baicalensis* have been used in traditional Chinese medicine, showing detoxifying, anti-inflammatory and antibacterial effects [3]. However, the mechanism of action of most extracts and essential oils has not been fully clarified [81]. For example, the antimicrobial activity of such drugs is provided by various plant secondary metabolites, among them are: geranyl acetate, eugenyl acetate, trans-Cinnamaldehyde, menthol, carvacrol, thymol, geraniol, eugenol, p-cimene, limonene, terpinene and carvone [82].

The mechanism of action of plant extracts and essential oils on a bacterial cell is probably associated with degradation of the cell wall, damage to the cytoplasmic membrane and its proteins, release of cellular contents, coagulation of the cytoplasm and destabilization of proton driving force [82]. Gram-positive bacteria are more susceptible to essential oils as compared to gram-negative ones, possibly because the latter have a thick layer of lipopolysaccharides in the outer membrane that covers the cell wall, limiting the diffusion of hydrophobic compounds [83].

In fact, many studies have confirmed the effectiveness of these plant derivatives against bacteria that cause inflammation of bovine mammary gland. For example, the scientists from Pakistan studied the antibacterial effect of *Allium sativum*, *Bunium persicum*, *Oryza sativa* and *Triticum aestivum* against strains of the most common pathogens of mastitis, such as *S. aureus*, *E. coli* and *K. pneumoniae*. It was found that all extracts significantly inhibit ($p < 0.01$;

$p < 0.05$) bacterial strain growth [40]. In another study, M. F. Cerioli et al. observed the inhibitory effect of *Minthostachys verticillata* essential oil and limonene on biofilm formation in *E. coli*, *Bacillus pumilus* and *Enterococcus faecium* strains isolated from cattle with signs of mammary gland inflammation. The results showed that the effect of essential oils is more apparent than limonene, which did not show bactericidal activity against *E. faecium* [41]. The Serbian scientists studied the antibacterial activity of *Thymus vulgaris* L., *Thymus serpyllum* L., *Origanum vulgare* L. and *Satureja montana* L. essential oils in the treatment of mastitis. For that, lactating cows of the experimental group received 15 mL of a drug containing essential oils into the mastitis-affected udder lobes. When comparing the total bacterial load in milk samples before and after treatment, it turned out that this drug effectively inhibited growth of *Staphylococcus* spp., *Streptococcus* spp., *Klebsiella* spp., *Proteus mirabilis*, *E. coli*, *S. uberis*, *Serratia marcenses*. The dominant compounds in the resulting product were thymol and carvacrol. The quantification of these two compounds in the evaluated biological samples showed that their withdrawal period is 24 hours [42].

However, there are some aspects that are considered as limiting the use of plant extracts and essential oils for the treatment of bovine mastitis. Thus, research should be aimed at finding industrial extraction methods, methods for converting plant extracts or essential oils into concentrated and homogeneous products and ways to use such drugs.

VACCINE PREVENTION

In many countries, the disease freedom of agricultural organizations is ensured by means of autogenic vaccines used mainly for the prevention of diseases caused by *S. aureus* and *Mycoplasma bovis*, and, to a lesser extent, by *S. uberis*. These vaccines are prepared based on isolates recovered on site from cows with mastitis, and then administered to the entire herd. In addition, commercial autogenic vaccines against mastitis are also available, for example, Bestvac® based on *S. aureus* strains (IDT, Germany) [84]. Mono- and polyvalent vaccines are also commercially manufactured. The vaccines against coliform mastitis available on the market include: 1) Enviracor™ J-5 contains the mutant strain J-5 *E. coli* (Zoetis, USA) and is administered subcutaneously three times (at drying off, in 4 weeks after drying off and within 2 weeks after calving); 2) J-VAC® *E. coli* contains bacterin-toxoid *E. coli* mutant strain J-5 (Merial, Germany) and is administered subcutaneously or intramuscularly twice (at drying off and in 2–4 weeks); 3) ENDOVAC-Dairy® is a bacterin toxoid derived from the mutant Re-17 *Salmonella typhimurium* (Endovac Animal Health LLC, USA), it provides protection against pathogens such as *E. coli*, *Salmonella*, *Pasteurella* and *Mannheimia*, is administered intramuscularly twice (at drying off and in 2–3 weeks). Vaccines effective against *S. aureus* are also available, for example Lysigin® (Boehringer Ingelheim, Germany), which is injected subcutaneously into the intramammary lymph node three times (4 weeks and 2 weeks prior to calving, with revaccination in 6 months).

Besides autogenic vaccines, inactivated ones are also used for mastitis prevention. The STARTVAC® multivalent vaccine (Hipra, Spain) contains *E. coli* (strain J-5) and *S. aureus* CP8 (strain SP 140) [85] and is administered intramuscularly thrice (45 days before calving, 10 days before calving, 62 days after the second vaccination). As for domestic products, there is MastitVak-EVA vaccine (ARRIAH, Vladimir) consisting of inactivated bacterial cells of *S. agalactiae*, *S. dysgalactiae*, *S. uberis*, two strains of *S. aureus*, *Staphylococcus hyicus* and two *E. coli* strains. For developing a primary immune background against the main clinically significant mastitis pathogens, it is recommended to vaccinate heifers starting from 20–22 weeks of age, and revaccinate after 2 weeks, followed by revaccination every 6 months.

Despite the fact that various commercial vaccines against mastitis are available, none of them provide complete protection, and moreover, are cost-effective [43]. There is evidence that the studies conducted in this respect did not reveal significant differences in mastitis occurrence and the somatic cell count in the milk of the control group cows and experimental groups of vaccinated animals [86]. The insufficient protective potential can be explained by many factors: age, health status, and different immune responses in individual animals depending on genetic and environmental conditions [3, 87, 88].

CONCLUSION

To sum it up, most of the literature data presented has shown the possibility of using new therapeutic approaches to overcome the limitations of traditional antibiotic-based therapy. However, for most of the alternative testing methods, only *in vitro* tests were conducted; additional, mainly *in vivo* tests, are not available yet, though they are critically important and necessary. The considered treatment methods probably will not be able to completely replace antibiotic therapy. The most rational solution would be to combine conventional antibiotic treatment schemes with new alternative approaches, this will reduce the duration of antibiotic use and the withdrawal period for milk, which, in turn, will increase productivity and reduce the likelihood of resistant bacterial strains. It should be considered that the prevention of bovine mastitis is achieved through improving the quality of life and conditions of animals, disinfection of udder nipples before and after milking, timely maintenance of milking machines, which are generally accepted measures to prevent the occurrence of new cases of mastitis.

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Rabies situation in the Moscow Oblast in 2011–2023 and the role of oral vaccination of wild carnivores against rabies

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ABSTRACT

Rabies is a zoonotic viral disease of all warm-blooded animals caused by a neurotropic virus, member of the *Lyssavirus* genus of the *Rhabdoviridae* family. About 59,000 people die from hydrophobia globally every year. According to the World Health Organization, the red fox (*Vulpes vulpes*) and the common raccoon dog (*Nyctereutes procyonoides*) are the main reservoirs and vectors among carnivores of the rabies virus in Europe. The paper describes animal rabies situation in 2011–2023 and the role of oral vaccination of wild carnivores against rabies in the Moscow Oblast. The region is a part of the Central Federal District and located in the center of the Russian plain bordering seven Oblasts (Tver, Smolensk, Kaluga, Tula, Ryazan, Vladimir and Yaroslavl Oblasts), which are also rabies infected. Notwithstanding the metropolis growth, the number of wild carnivores in the Moscow Oblast remains high. Comprehensive preventive measures to control the population of the wild carnivores are taken to stabilize the rabies situation and decrease the incidence, innovative achievements in laboratory diagnosis are introduced, population immunity of wild carnivores by oral vaccination is improved and the epidemic situation in neighboring regions is analyzed. In 2017 the systemic, consistent and thoroughly organized campaign was started – the oral vaccines were distributed by light aircrafts. The research revealed the correlation between the decrease in annual number of reported rabies cases and increase in the amounts of oral vaccines distributed. The use of controlling devices (camera traps) confirmed that oral rabies vaccines are consumed by the target animals (red foxes). The onward systemic, methodical approach to rabies prevention will mitigate the risks of rabies occurrence in the Moscow Oblast.

Keywords: rabies virus, red fox, common raccoon dog, oral vaccination, epidemic situation, Moscow Oblast

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Эпизоотическая ситуация по бешенству на территории Московской области (2011–2023 гг.) и роль оральной вакцинации диких плотоядных

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РЕЗЮМЕ

Бешенство – зоонозное вирусное заболевание теплокровных животных, возбудителем которого является нейротропный вирус рода *Lyssavirus* семейства *Rhabdoviridae*. Ежегодно в мире от гидрофобии погибает около 59 000 человек. В Европе, по данным Всемирной организации здравоохранения, основными видами диких плотоядных, которые поддерживают природные очаги бешенства, являются лиса (*Vulpes vulpes*) и енотовидная собака (*Nyctereutes procyonoides*). В статье представлена эпизоотическая картина по бешенству животных (2011–2023 гг.), проанализирована роль оральной вакцинации диких плотоядных в Московской области. Регион входит в состав Центрального федерального округа, расположен в центре Русской равнины и граничит

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с семью областями: Тверской, Смоленской, Калужской, Тульской, Рязанской, Владимирской, Ярославской, – которые также являются неблагополучными по бешенству животных. Несмотря на урбанизацию мегаполиса, численность диких плотоядных животных в Московской области остается высокой. В регионе проводится системная профилактическая работа по контролю численности диких плотоядных животных, стабилизации эпизоотической ситуации и уменьшению случаев бешенства, внедряются передовые научные разработки в области лабораторной диагностики, повышения популяционного иммунитета среди диких плотоядных животных путем оральной вакцинации против бешенства, анализируется эпизоотическая ситуация в сопредельных областях. С 2017 г. в Московской области началась системная, планомерная, тщательно организованная кампания – с помощью средств малой авиации стала проводиться тотальная раскладка оральной вакцины. Исследования выявили корреляцию между снижением ежегодного числа регистрируемых случаев бешенства и увеличением объемов использования оральной вакцины. Применение средств внедренного объективного контроля (фотоловушек) подтвердило поедание оральной антирабической вакцины целевыми видами животных (лисицами). Дальнейший системный, методичный подход к профилактике бешенства снизит риски возникновения вспышек этого заболевания в Московской области.

Ключевые слова: вирус бешенства, красная лисица, енотовидная собака, оральная вакцинация, эпизоотическая ситуация, Московская область

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INTRODUCTION

Rabies is an infectious disease of mammalian animals caused by a neurotropic virus (genus *Lyssavirus*, family *Rhabdoviridae*). Rabies virus (RABV), as a typical lyssavirus occurs in different parts of the globe, has reservoir hosts, which maintain the viral circulation [1]. From the primary reservoir host, the virus is transmitted to other susceptible animals and to humans (Fig. 1). In Europe, such animals are the red fox (*Vulpes vulpes*) and the raccoon dog (*Nyctereutes procyonoides*) [2].

Lyssavirus species affect only mammals. The evolution of lyssaviruses is associated with the animal species in which the virus can maintain an independent cycle of development. These are a wide range of mammalian species within the *Carnivora* and *Chiroptera* orders. They are spread on various continents of the globe, excluding Antarctica. It is generally accepted that bats are the true primary reservoir hosts of almost all lyssaviruses. However, unlike all other lyssaviruses, rabies viruses (RABVs) as the typical species for lyssaviruses have established multiple independent transmission cycles in a broad range of carnivore host reservoirs. Typical carnivore host reservoirs for RABV are dogs, jackals, raccoon dogs, coyotes, skunks; in Eurasian and American arctic and subarctic regions is the arctic fox (*Alopex lagopus*) [1, 3].

In the Russian Federation, rabies cases are reported in many regions of the country, including the Moscow Oblast [4, 5], which is part of the Central Federal District (CFD) and located in the center of the Russian Plain. The region is adjacent to the Tver, Smolensk, Kaluga, Tula, Ryazan, Vladimir, and Yaroslavl Oblasts, which are also rabies infected. The fauna of the CFD regions includes reservoir hosts of the rabies virus that maintain the sylvatic cycle of the disease. The main role in this process is played by the red fox (*Vulpes vulpes*), which is one of the most common predatory mammals among canines inhabiting the Moscow Oblast. It is a medium-sized carnivorous various shaded red predator, which dwells in burrows and sees well in the dark. The diet includes mainly small rodents [6, 7, 8, 9, 10, 11]. The density of the fox population

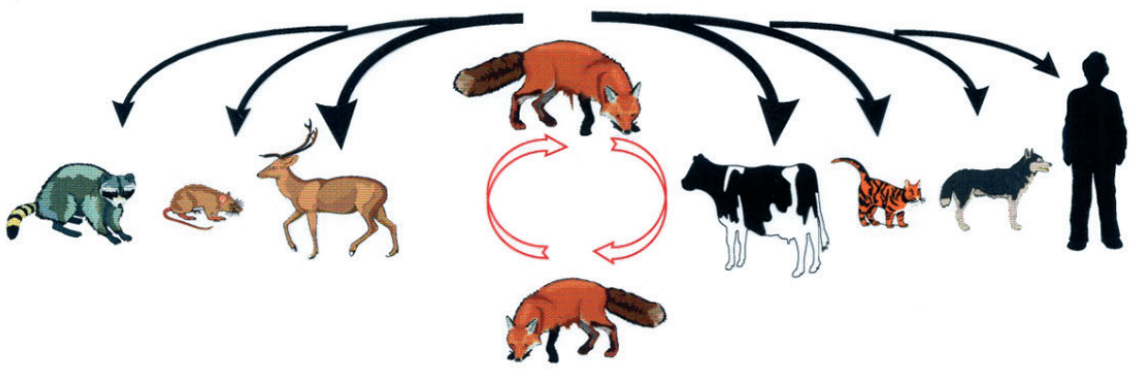

Fig. 1. Circulation and transmission of the rabies virus explained by the example of the red fox, Vulpes vulpes (https://www.who-rabies-bulletin.org/sites/default/files/epi_1.jpg)

per 1,000 hectares in the Moscow Oblast varies depending on the area. Fox harvesting in 2010–2021 was not regular due to the lack of demand for fox pelts [11].

The Moscow Oblast surrounds the city of Moscow and is a region in which the rabies situation is under special control. Despite the high urbanization levels in the megalopolis, the number of wild carnivores in the Moscow Oblast remains high. The geographical position in the central part of the East European Plain with favorable climatic and landscape conditions for the habitat of carnivores contribute to that [12]. An increase in the fox population density, in our opinion, is the main risk factor for the maintenance of rabies sylvatic cycle and incidence in the Moscow Oblast. This is confirmed by the data published by N. I. Osipova [13].

Rabies remains a global threat. It causes great economic damage to agriculture. The rabies virus accumulates in the saliva and brain of infected animals and is transmitted through biting and licking. The transmission through contacts does not evoke any explosive outbreaks of rabies and its rapid spread, as it happens with highly contagious diseases, for example, foot-and-mouth disease. Many species of farm animals are considered to be dead end hosts (cattle, sheep, goats, pigs, horses) in the rabies epizootic chain. They usually die when infected, although all diseased animals and animals during the incubation period are the source of the virus, and dead animals and virus contaminated environmental objects become transmission factors.

Low ambient temperatures are favourable for the preservation of the virus virulence. In this regard, the carcasses of animals killed by rabies pose a real danger, since the virus remains alive in them for 2–3 weeks, and at subzero temperatures for several months [14, 15].

It is difficult to overestimate the social significance of the disease, since rabies poses a real threat to humans. Human deaths from rabies are also reported in the Russian Federation. This is the case when an individual does not seek medical assistance after an animal bite, scratch or licking. The sylvatic rabies contributes to the maintenance of rabies virus circulation in the Russian Federation, therefore, the risk of human infection is always high. Rabies cases are reported more often in spring and summer. A rabid animal causes typical injuries to humans. Especially dangerous targets are head, face, and fingers. The exposed human shall be immunized against rabies as soon as possible. For specific prophylaxis, COCAV rabies vaccine is used. For severe cases, after multiple bites, combined therapy scheme using the COCAV vaccine and specific gamma globulin has been developed. In order to prevent rabies in humans, it is necessary to raise awareness among public through mass-media [16]. About 59,000 humans die from rabies every year in the world [17].

All this identifies the challenges faced by the Moscow Oblast Veterinary Service when developing animal rabies control plans. In addition to constantly changing economic environment of the metropolis, it is important to take into account the biology and ethology, firstly, of foxes and raccoon dogs, changes in their food supplies and population density. The threshold value of the fox population density when sylvatic rabies is reported is more than one individual per 1 km^2 [18].

Advanced scientific developments in laboratory diagnosis, aimed at improvement of population immunity among wild carnivores through oral rabies vaccination are introduced in the Moscow Oblast, and the disease situation in the neighboring regions is analyzed.

Oral vaccination of wild carnivores remains a recognized method of rabies prevention in the complex of disease control measures taken by the veterinary services both in our country and abroad [19, 20].

The aim of the research was an in-depth study of the animal rabies situation in the Moscow Oblast, as well as an assessment of the oral immunization role for wild carnivores.

MATERIALS AND METHODS

Data from statistical reports of the Moscow Oblast State Veterinary Service for the period from 2011 to 2023 were used for the work. The data were processed by descriptive and evaluative epidemiological and statistical methods. To determine the territorial-geographical location of rabies cases search engines, like Google Earth Pro and Yandex, were used.

The final diagnosis of rabies was made after confirmation by laboratory tests in accordance with approved GOST 26075-2013 "Animals. Methods of Laboratory Diagnostic of Rabies"[1] and "Recommended practice for the diagnosis of animal rabies by immunofluorescence"[2].

Tetracycline in teeth and bones of carnivorous animals was determined in accordance with the "Guidelines for the detection of tetracyclines by fluorescence method in animal teeth and bones to control the oral rabies vaccine consumption"[3] and the recommendations of A. M. Gulyukin [21]. For sample preparation, a Buehler low-speed precision cutter (USA) was used to determine the tetracycline marker by making 1–2 mm thick cuts of teeth and jaws of wild carnivores.

For the assessment of epidemiological surveillance results, the method of retrospective epidemiological analysis was used.

RESULTS AND DISCUSSION

The effectiveness of the disease control comprises a whole range of measures developed by the Moscow Oblast Veterinary Service, which are adjusted to concrete conditions:

– strict recording of susceptible animals in order to implement rabies vaccination plans among pets and livestock, zoo animals, as well as to comply with the regulatory requirements for appropriate and humane keeping of domestic carnivores;

– interaction with employees of hunting farms to control the fox density;

– oral vaccination of wild carnivores against rabies;

– short-term and long-term forecasting;

– awareness raising campaigns among public with regards to the rabies dangers and prevention.

Rabies prevention and control with an ultimate goal of its eradication also involves the development and implementation of a constant monitoring of this infectious

[1] https://docs.cntd.ru/document/1200104625 (in Russ.)

[2] Sukharkov A. Yu., Eremina A. G., Nazarov N. A., Egorov A. A., Metlin A. E., Shulpin M. I. Recommended practice for the diagnosis of animal rabies by immunofluorescence: MR 33-16. Vladimir: FGBI "ARRIAH", 2016. 14 p. (in Russ.)

[3] MG 36-16 Guidelines for the detection of tetracyclines by fluorescence method in animal teeth and bones to control the oral rabies vaccine consumption. Vladimir: FGBI "ARRIAH", 2016. 11 p. (in Russ.)

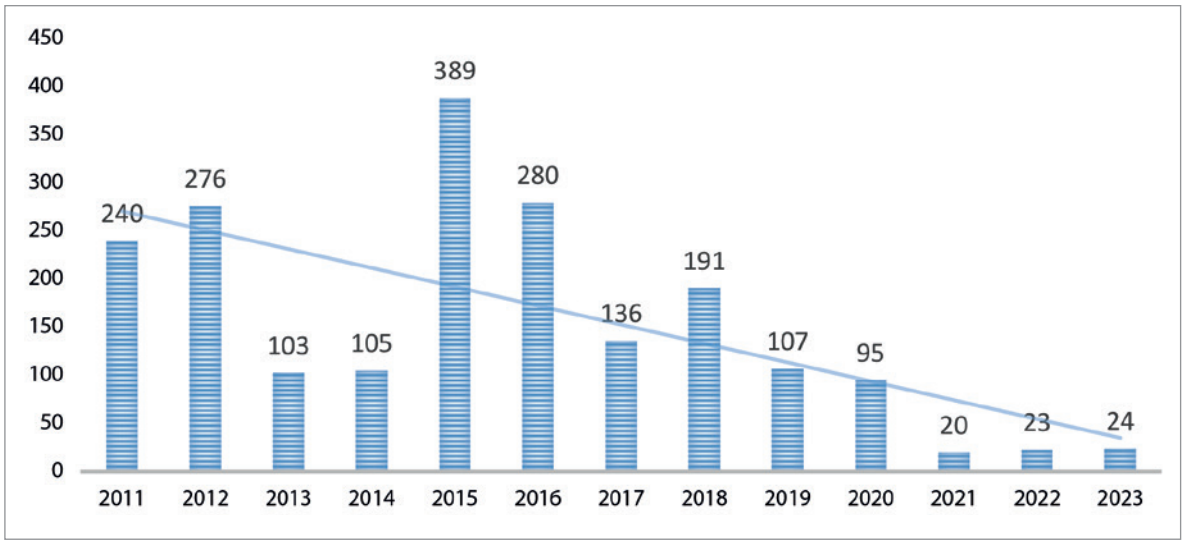

Fig. 2. Animal rabies incidence reported in the Moscow Oblast in 2011–2023 and its trend

disease in a certain area over a certain period of time, i.e. epidemiological surveillance and control.

It is important to identify possible carriers, to recognize the manifestation (presence) of the disease in a healthy population as early as possible, to prove the absence of rabies in susceptible animal subpopulations, and to establish the disease trend. To do this, well-known strategies are used: monitoring, screening, examination, observation, etc.

Every year since 2011, state epidemiological monitoring plans, covering rabies tests, have been implemented in the Moscow Oblast. They are implemented by the institutions subordinate to the Rosselkhoznadzor. In addition, diagnostic test plans are implemented at the level of the Russian Federation Subject, which also cover testing for rabies. According to the regulatory documents of the Russian Federation, data on tests performed shall be provided to the relevant organizations using the approved templates. Test reports shall be entered into the Rosselkhoznadzor information systems "Assol" and "Vesta".

This system is used to detect the disease, prevent and reduce morbidity, and ultimately eradicate rabies in the designated areas.

As can be seen in Figure 2, rabies was reported annually in the Moscow Oblast in 2011–2023. The rises and falls in the disease manifestation over the years are clearly visible, which confirms the rabies cycle nature. The beginning of the observation period coincides with an increase in the total number of reported cases (2011–2012), followed by a decline and a new rise. The highest rabies incidence was reported in 2015. In general, the trend is decreasing.

The species structure of animals involved in the rabies epizootic process is shown in Figure 3. The highest incidence over the entire observation period was reported in wild carnivores (65%), followed by domestic carnivores (33%) and farm animals (2%). It should be noted that the objectivity of reports on animal rabies has increased since "Sirano" system was introduced in our country.

A relatively high incidence of rabies among carnivores during the observation period suggests the sylvatic cycle of the disease, when the vectors, in particular diseased foxes, can carry the virus to potential victims or can transmit the virus to susceptible animals (stray cats, dogs and farm animals). Monitoring tests have shown that the number of stray domestic carnivores, as a rule, increases in autumn, when the summer season is over. This

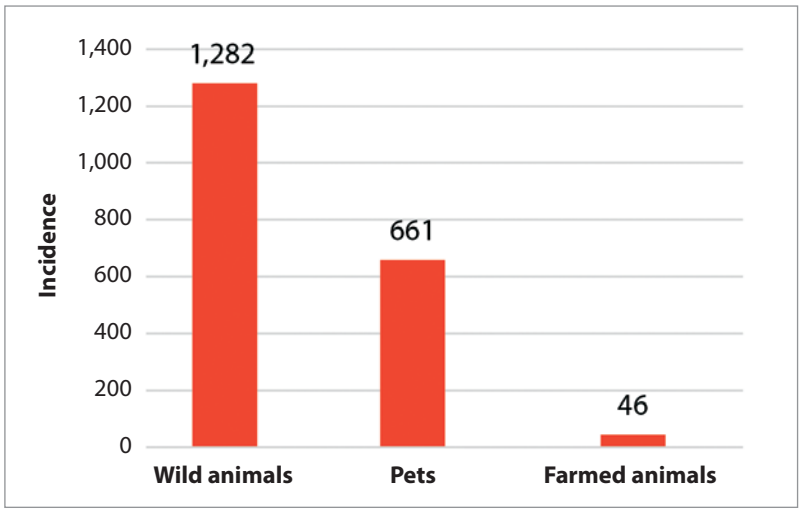

Fig. 3. Species structure of animal rabies in the Moscow Oblast in 2011–2023

Fig. 4. Light aircraft for oral vaccine distribution

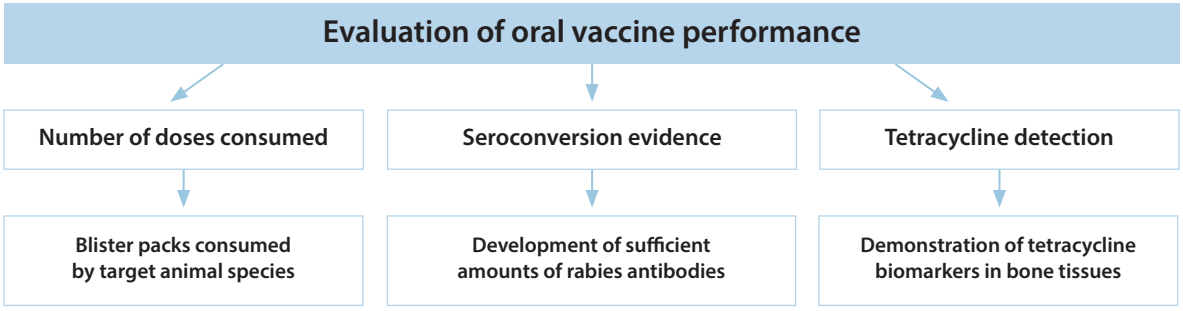

Fig. 5. Evaluation of oral vaccination results

peculiarity was highlighted by B. L. Cherkassky [22]. It is necessary to implement awareness raising campaigns among the public in the urban territories, where special conditions for the rabies virus circulation are created.

Stray animals bring additional risks of rabies occurrence and increase in its incidence. To solve the social and economic problem of stray animals in the Moscow Oblast a management system has been created to improve the coordination and interaction of services, directly or indirectly responsible for trapping and keeping of stray animals. This will increase the effectiveness of control and measures taken to regulate the number of stray animals.

Due to the fact that wild carnivores are the main rabies reservoir, oral vaccination has been initiated in the region. The first field trials conducted in Switzerland in the 70s of XX century, showed that oral immunization is an effective method of animal rabies control. The possibility of using a live attenuated strain of rabies virus embedded into a special bait attractive for wild carnivores was demonstrated. The attenuated virus penetrates the lymphoid tissue through the oral cavity of the animal, activates the immune response inducing resistance to the infection with the virulent virus. Oral immunization is currently a highly effective method of the disease control. The modern strategy of rabies control invariably includes specific prevention of the disease among domestic carnivores [14]. The experience of the European Union countries, the USA, and Canada has shown that the consistent long-term use of oral rabies vaccines in wildlife effectively reduces the incidence, until the disease is eradicated.

The main purpose of oral immunization is to induce and strengthen specific immunity in susceptible wild carnivores. The presence of specific virus neutralizing antibodies in the sera of the target vaccinated animals in a titer of ≥ 0.5 IU/cm^3 provides sufficient protection in the target animal species [23, 24].

Rabivak-O/333 (vaccine for oral immunization of wild carnivores against rabies, Pokrovsky Plant of Biological Products, Russia) and Rabistav (vaccine against rabies of wild carnivores, Stavropol Bioplant, Russia), registered and authorized in the Russian Federation, were used in the Moscow Oblast. When handling the vaccines, all rules prescribed in the instructions for their use were carefully followed.

Oral rabies vaccines are constructed as follows: a blister or capsule with a viral suspension is embedded into a bait in the shape of a rectangular block, weighing 25–55 g.

At the beginning, when the oral vaccination was introduced, the vaccine was distributed using two methods: manual baiting in the territory of the Moscow Oblast municipalities at the rate of 25–30 doses of the vaccine per 1 km^2, and aerial distribution in hard-to-reach areas 2 times a year. Personal safety measures were obligatorily observed during the distribution process.

Since 2017, light aircrafts have been used in the Moscow Oblast to distribute oral vaccines (Fig. 4), in compliance with the international requirements. The distribution pattern is based on GPS mapping. Before the vaccination campaign, the area was mapped; flight charts were made to depict the planned routes for the vaccine distribution, zones to be covered by light aircraft were defined and approved; controlled forest areas were highlighted.

Fig. 6. Camera trap "Filin 120"

Fig. 7. Approaching and consuming of vaccine baits by foxes in the controlled area

Spring vaccination campaign was conducted in late March, April, and early May (depending on weather conditions). The second vaccination was performed in autumn, in September – October. Due to the fact that after spring vaccination, fox cubs have colostral immunity, the third vaccination was conducted in June or early July.

The vaccination campaign effectiveness was evaluated (Fig. 5), which included visual determination of the bait up-take at the control sites, sampling and their laboratory testing to determine the tetracycline marker in teeth and bones and the level of rabies antibodies in wild carnivores.

The active use of the oral vaccines against rabies among wild carnivores required correction of the vaccination effectiveness evaluation, search for and introduction of new methods. Therefore, we have studied the possibility of using camera traps. The camera trap is a fully automatic camera with GSM functions, which is disguised from animals using a special case. "Filin 120" camera trap was used for this work (Fig. 6). It is triggered by a motion sensor and automatically captures images or videos when an animal appears in the controlled area at a distance of up to 20 m.

Thus, for the first time in Russia, a remote method for rabies oral vaccine up-take control and efficacy evaluation was used in the Moscow Oblast. The camera trap automatically sends images to a mobile phone using a GSM/GPRS network. The MMS make it possible to receive 1–99 images, which are processed by a computer (Fig. 7).

The camera trap data showed that all distributed vaccine baits were willingly consumed during the first two days by the main target animal species – the fox. The up-take rate at the controlled areas was calculated and was equal to 70–90%.

The vaccine uptake is also controlled by the tetracycline marker. When consumed, tetracyline binds to growing bones, in particular, to tooth tissue, and can be detected by the fluorescence method in teeth or mandible bones [25].

The analysis of the rabies epizootic process in 2013–2015, conducted by M. I. Gulyukin and A. A. Shabeykin [26], showed that there was an increase in the disease incidence in most of the European territory of Russia. The oral vaccination campaign carried out in the country did not give the expected effect, except in some isolated regions. According to A. A. Shabeykin [27], the sylvatic nature

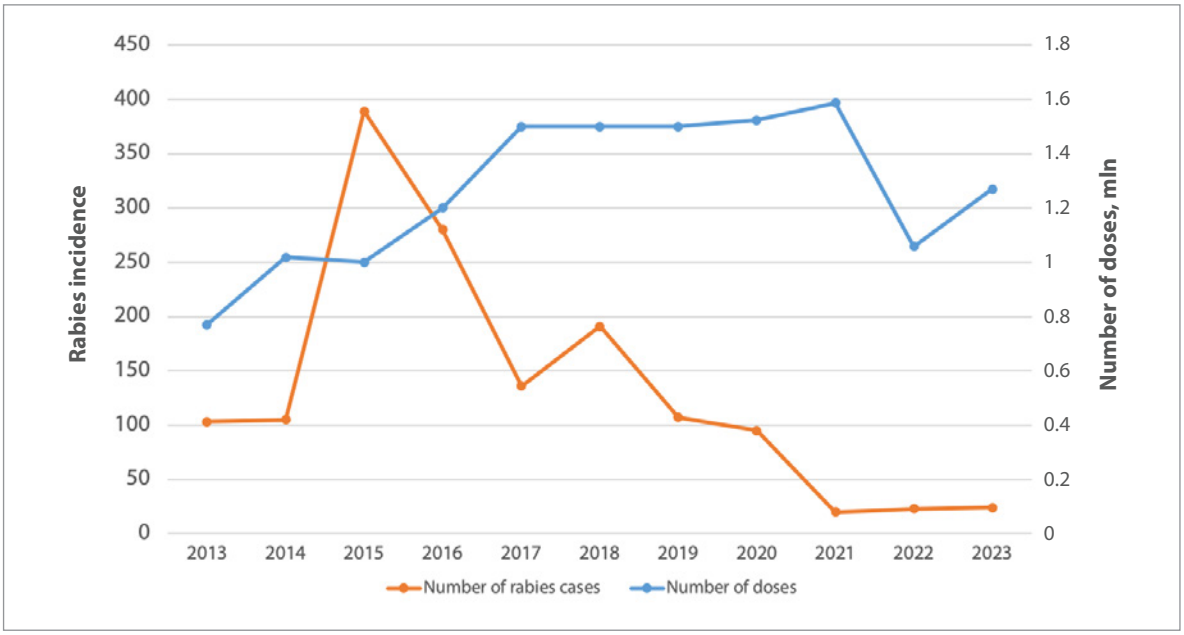

Fig. 8. Rabies incidence and number of the used oral vaccine doses in the Moscow Oblast in 2013–2023

of rabies in the Russian Federation underlies its spread geography, seasonality, outbreak periodicity and species involved in the epizootic process, and parameters of species- and spatiotemporal manifestations of the epizootic process are subject to constant changes. The large regions of the country, the size of the disease distribution area, the variety of geographical conditions, and the renewal of the reservoir animal population are the factors that significantly complicate the task of wild carnivore vaccination in Russia. This dictates the need to further improve the strategy of oral rabies vaccination.

Spatial analysis of the data and digital models of the epizootic processes created by A. A. Shabeykin [28] made it possible to determine rabies epizootic development patterns with regard to natural zones and provinces of the Russian Federation. In the conditions of mixed forest biomes, there is a shift towards higher number of rabies cases reported among wild carnivores. Rabies incidence in raccoon dogs is at its maximum level in forest biomes, where this animal species is most likely an additional biological reservoir of the rabies virus.

In the Moscow Oblast, too, despite the ongoing oral vaccination of wild carnivores in 2013, 2014, 2015 (0.770; 1.018; 1 million doses of vaccine were used, respectively), the number of rabies cases increased, reaching its maximum (389 cases) by 2015.

Since the oral vaccination method has proved its effectiveness in many countries of the world, a systematic, consistent and thoroughly arranged vaccination campaign was launched in the region. "Recommended practice for rabies oral vaccination of carnivores in the Moscow Oblast"[4] was developed and approved by the Main Veterinary Department of the Moscow Oblast and the annual number of the vaccines used increased: from 1.2 million doses in 2016 up to 1.587 million doses in 2021. The number of rabies cases in the wild fauna started decreasing. A slight increase in the rabies incidence in 2018 (191 cases) was replaced by a significant decrease by 2021 (20 cases), after which a kind of plateau was formed by 2023, when 24 cases of rabies were reported (Fig. 8).

A retrospective analysis of the rabies situation in the Moscow Oblast showed that in 2011–2023 there were three rises and falls in the intensity of the rabies epizootic process. The peaks occurred in 2012, 2015 and 2018. Despite the subsequent sharp decrease in the reported rabies incidence in the region, the disease is persisting in wild fauna, which suggests the presence of sylvatic rabies.

CONCLUSION

The rabies situation in the Moscow Oblast at the beginning of the period under review (from 2011 to 2015) can be considered tense. It was characterized by the sylvatic cycle of rabies and the epizootic periodicity [29]. The reservoirs are mainly red foxes. The subsequent implementation of the set of disease control measures, including intensive annual oral vaccination of wild carnivores in compliance with strict recommendations for its use, has reduced the reported incidence and the intensity of the epizootic process in the region. The Moscow Oblast Veterinary Service confirmed the high importance of constant monitoring of all handling of rabies-susceptible animals.

It was shown that a decrease in the number of annually reported rabies cases correlated with an increase in oral vaccine doses used. The data recording devices (camera traps) confirmed the up-take of oral rabies vaccine by target animal species (foxes).

The introduction of advanced scientific developments in laboratory diagnostics, enhancement of population immunity among wild carnivores through oral vaccination and analysis of the animal disease situation in neighboring regions have improved the animal rabies situation in the Moscow Oblast.

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Development and validation of highly sensitive multiplex real-time RT-PCR assay for detection of classical swine fever virus genome

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ABSTRACT

Classical swine fever (CSF) remains a challenge for pig farming industry all over the world despite the measures taken. The last CSF case in the Russian Federation was reported in 2020, however, the threat of the disease emerging still persists. A set of anti-epidemic measures including mainly preventive vaccination and annual diagnostic monitoring using molecular-genetic and serological methods is required for CSF virus introduction prevention and rapid eradication of potential disease outbreaks. Therefore, a real-time reverse transcription-polymerase chain reaction using an internal control sample has been developed. Therefore, a real-time reverse transcription-polymerase chain reaction using an internal control sample has been developed. Modified primers (locked nucleic acids containing conformationally blocked nucleosides) providing a higher affinity to the DNA matrix and physicochemical stability and a FAM-labeled TaqMan probe were selected for 5'-untranslated region of the genome. The following validation parameters were defined: accuracy, repeatability, reproducibility, specificity and sensitivity. For comparative analysis of the developed assay sensitivity, swabs, samples of organs and tissues collected from pigs experimentally infected with an epizootic strain of the classical swine fever virus (spleen, kidney, liver, blood, lymph nodes, rectal and oral smears), animal-contaminated feed and virus-containing material with known virus titres were also tested in parallel with coded test systems No. x1 and x2. The developed assay was shown to have 100% diagnostic sensitivity and detection limit of 0.23 lg $CCID_{50}/cm^3$. Therewith, the results of analysis of test systems No. x1, x2 based on above parameters were lower that could give rise to false positive real-time RT-PCR results and incorrect diagnosis. Thus, described assay can be used for extensive monitoring of classical swine fever in the Russian Federation.

Keywords: classical swine fever, real-time RT-PCR, internal control sample, diagnostic sensitivity, analytical sensitivity

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Разработка и валидация высокочувствительного метода мультиплексной ОТ-ПЦР-РВ для обнаружения генома вируса классической чумы свиней

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РЕЗЮМЕ

Проблема классической чумы свиней в свиноводстве по-прежнему остается актуальной во всем в мире, несмотря на принимаемые меры. Последний случай данного заболевания в Российской Федерации регистрировали в 2020 г., однако сохраняется угроза эмерджентного возникновения болезни.

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Для предотвращения заноса вируса классической чумы свиней и быстрой ликвидации потенциально возможных вспышек необходимо проведение комплекса противоэпизоотических мероприятий, преимущественно включающих вакцинопрофилактику и ежегодный диагностический мониторинг на основе молекулярно-генетических и серологических исследований. В связи с этим разработан метод полимеразной цепной реакции с обратной транскрипцией в режиме реального времени с использованием внутреннего контрольного образца. Праймеры в модификации Locked Nucleic Acid (конформационно блокированных нуклеозидов), обеспечивающие более высокий уровень аффинности к ДНК-матрице и физико-химической стабильности, и FAM-меченый TaqMan-зонд были подобраны к 5′-нетранслируемой области генома. Также определены валидационные показатели: правильность, сходимость, воспроизводимость, специфичность и чувствительность. С целью сравнительного анализа чувствительности параллельно тестировались зашифрованными тест-системами № х1, х2 образцы смывов, органов и тканей, полученных от свиней, экспериментально зараженных эпизоотическим штаммом вируса классической чумы свиней (селезенка, почка, печень, кровь, лимфатические узлы, ректальные и оральные мазки), корма, контаминированного животными, и вируссодержащего материала с известными титрами. Показаны 100%-я диагностическая чувствительность и предел детекции в 0,23 lg ККИД$_{50}$/см^3 разработанного метода. При этом показатели тест-систем № х1 и х2 были ниже, что может приводить к ложноотрицательным результатам полимеразной цепной реакции с обратной транскрипцией в режиме реального времени (ОТ-ПЦР-РВ) и влиять на недостоверную постановку диагноза. Таким образом, представленный метод может использоваться при проведении широкомасштабного мониторинга классической чумы свиней в Российской Федерации.

Ключевые слова: классическая чума свиней, ОТ-ПЦР-РВ, внутренний контрольный образец, диагностическая чувствительность, аналитическая чувствительность

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INTRODUCTION

Classical swine fever (CSF, *Pestis suum*) is one of the major viral diseases having a significant impact on pig farming and wild boar hunting [1].

The CSF etiological agent is a *Pestivirus C* with a positive-sense, single-stranded RNA genome of 12.3 kb in length of the genus *Pestivirus*, family *Flaviviridae* [2]. RNA molecule contains 2 nontranslated regions (5′-NTR and 3′-NTR), as well as one open reading frame coding for 13 proteins (4 structural and 9 nonstructural proteins) [3].

According to the recommendations of the World Organization for Animal Health (WOAH) classical swine fever is subject to notification [4]. And despite the fact that CSF was eradicated in the European countries and in Russia, where the last outbreak in domestic pigs was reported in 2019 and the last outbreak in wild boars was reported in 2020, the threat of this transboundary disease introduction still persists, that requires systematic disease monitoring [5]. Due to the lack of reliable data on the number of samples tested within passive serological monitoring, it is difficult to reliably prove absence of virulent CSFV circulation in wild boar population in Russia [6].

Currently, the polymerase chain reaction (PCR) assay is widely used as one of the most rapid, specific and sensitive molecular biological methods for the pathogen genetic material detection [7]. However, the classical PCR assay with electrophoretic detection in agarose gel is a time- and labour-consuming method posing a high risk of cross-contamination [8]. The real-time multiplex reverse transcription polymerase chain reaction (real-time RT-PCR) with an internal control sample (ICS) minimizing unreliable results is the most suitable for CSF diagnosis, including screening and monitoring [9].

TaqMan probes enabling real-time hybridization-fluorescence detection of PCR products and being the most practical and reliable ones for pestivirus infection diagnosis are used in some assays [10]. Most real-time RT-PCR-based test systems described earlier for the CSFV genome detection amplify a fragment of 5′-nontranslated region (5′-NTR) and demonstrate sufficient sensitivity and specificity [11]. Modification of oligonucleotides in locked nucleic acids (LNA) increases primer affinity to the target DNA and provides for physicochemical stability [12]

Exogenous ICS allows for avoiding false negative results due to errors both at the stage of sample preparation (nucleic acid extraction) and at the stage of the target fragment amplification [13].

The study was aimed at development and validation of highly sensitive, specific and reproducible multiplex real-time RT-PCR assay enabling diagnosis of all CSFV subgenotypes circulating in the Russian Federation territory (1.1, 1.2, 2.1, 2.3) in the period from 1982 to 2020 [5, 14]. Such assay should have characteristics meeting all the requirements for a real-time PCR-based test system and be widely applicable in monitoring for the infection diagnosis [15].

MATERIALS AND METHODS

Viruses and bacteria. The following CSFV strains were used for the assay development: reference Shimen strain, 684 strain, 719 strain (subgenotype 1.1), vaccine SK strain (subgenotype 1.2), CSF Amur 19-10/WB-12555 strain and CSF Tigrovoe 16/WB-634 (subgenotype 2.1), 275 strain (subgenotype 2.2), 368, 870, 843 strains (subgenotype 2.3) and CSFV 589, 924, 925, 929, 917, 918, 920, 926, 927, 930 strains with unidentified virus genotype isolated in the period of 1982–2020.

The following heterologous porcine disease agents were used for determination of the assay analytical specificity: vaccine VK-DEP strain of Aujeszky's disease virus, Irkutsky 2007 strain of American porcine reproductive and respiratory syndrome virus (PRRSV), reference Mozambique-78 strain of African swine fever virus (ASFV) genotype V; Chelyabinsk 2021 isolate of bovine viral diarrhea virus (BVDV) genotype II, field isolate of swine erysipelas agent (*Erysipelothrix rhusiopathiae*). The biological pathogens were obtained in the form of freeze-dried material from the State Microorganism Strain Collection and working microorganism collection of the Federal Centre for Animal Health.

Animals. Piglets at the age of 2–2.5 months weighing 10–15 kg and obtained from CSF-free farms located in the Vladimir Oblast were used for primary cell culture preparation. The piglets were euthanized and testicular explants were collected in accordance with the requirements of 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.

Cultivation. CSFV was propagated in the initially trypsinized swine testicle (ST) cell culture cultivated in Eagle-MEM medium prepared according to the Federal Centre for Animal Health procedure and supplemented with 10% bovine fetal serum and 50 μg/cm^3 of gentamicin sulphate [16]. The virus reproduction was identified with real-time RT-PCR according with the methodical guidelines[1].

Internal control sample (ICS). MS2, RNA-containing bacteriophage belonging to *Leviviridae* family and pathogenic for *Escherichia coli*, was selected as an ICS [17].

Designing of primers and probes. cDNA nucleotide sequences of different CSFV subgenotypes imported from the GenBank database were aligned and subjected to comparative molecular-genetic analysis using Bioedit v7.2.5 and NCBI: Nucleotide BLAST software. Conserved segments of CSFV genome served as a criterion for selection of optimal primers. Primers and probe for amplification and hybridization of ICS fragment were selected based on the literature data [18]. Synthesis of oligonucleotides was performed at the Syntol company (Russia).

Extraction of nucleic acids. CSFV RNA was extracted from the virus-containing ST cell culture suspension, genomes of heterologous viruses and bacteria were extracted from freeze-dried materials with nucleosorption method using RIBO-sorb reagent kit for DNA/RNA extraction from biological materials (Central Research Institute of Epidemiology, Federal Service for Surveillance on Consumer Rights Protection and Human Wellbeing, Russia) in accordance with the manufacturer's instructuctions[2].

Real-time RT-PCR procedure. PCR master mix produced by the Eurogene (Russia) contained the following components: OneTube RT-PCR TaqMan kit consisting of OneTube RT-PCRmix, TM-MMLV revertase, nuclease-free water. All PCR stages (reverse transcription, amplification and fluorescence-hybridization detection) were performed in automatic Rotor-Gene Q thermocycler using provided software (QIAGEN, Germany).

Positive control sample (PCS). Vaccine SK strain of CSFV (virus titer – 3.5 lg $CCID_{50}$/cm^3) in the form of freeze-dried material dissolved in 4.0 cm^3 of saline solution and thermally inactivated by heating for 60 minutes at +60 °C was used as a positive control. Tests for inactivation completeness were carried out by three blind passages in ST cell culture according to the methodical guidelines[1].

Negative control sample (NCS). Nuclease-free water produced by the Eurogene (Russia) was used as a negative control.

Validation. Validation parameters were determined in accordance with the recommended guidelines for publication of the quantitative real-time PCR development outcomes (MIQE: Minimum Information for Publication of Quantitative Real-Time PCR Experiments) [19]. To determine accuracy, repeatability and reproducibility CSFV 719 strain-positive sample was analyzed in 6 repeated reactions, in 3 parallel tests performed by the same operator within one day and in 3 parallel tests performed by two operators within 3 days. Analytical sensitivity (detection limit) was estimated using 10-fold dilutions of CSFV 719, 684, Shimen strain with known titer. Detection limit was expressed as minimal virus titer (lg $CCID_{50}$/cm^3) detected by the assay under validation. Amplification efficiency was calculated according to the formula:

$$E = (10^{1/slope} - 1) \times 100\%,$$

where slope – the slope value of the linear region of the curve constructed using the Ct values plotted on a logarithmic scale according to cDNA matrix concentration.

The real-time RT-PCR assay was tested for its analytical specificity using known CSFV RNA-negative samples containing extracted genomes of heterologous viruses and bacteria as well as using CSFV strains of different subgenotypes. To tests the assay for its diagnostic sensitivity a panel of 27 true positive samples (spleen, liver, kidney, blood, lymph nodes, muscle, oral and rectal swabs) collected from the pigs experimentally infected with epizootic CSFV strain at different stages of the infection process as well as contaminated feed samples was prepared. The assay was tested for its diagnostic specificity by examination of 27 known negative samples of complete mixed feed for pigs, 10% spleen, liver, lymph node suspensions, porcine meat products, whole blood, oral and rectal swabs that were free from CSF and were prepared at the Federal Centre for Animal Health Reference Laboratory for ASF for official animal disease monitoring implementation and for commercial tests of samples from pig holdings located in the European part of the Russian Federation. The assay

[1] Kolbin I. S., Vlasova N. N., Igolkin A. S., Elsukova A. A., Gavrilova V. L., Puzankova O. S. Methodical guidelines for isolation of classical swine fever virus with real-time polymerase chain reaction with fluorescent hybridization probe for the product detection in primary cell cultures (porcine spleen, porcine bone marrow, porcine kidney, lamb testicle, swine testicle) approved by the Federal Centre for Animal Health on 14 September 2021, No. 42-21. Vladimir: Federal Centre for Animal Health, 2021. 56 p. (in Russ.)

[2] Instruction on use of RIBO-sorb reagent kit for DNA/RNA extraction from clinical materials: approved by the Order No. 1337-Pr/09 of the Federal Service for Surveillance in Health Care of 20 February 2009. https://www.amplisens.ru/upload/iblock/259/RIBO-sorb.pdf (in Russ.)

was tested for its diagnostic and analytical sensitivity in comparison with coded Russian test-systems No. x1, x2 by parallel testing of the samples. The coded test systems were used according to their manufacturers' instructions. The selected test systems are the most commonly used in Russia for the CSF diagnosis and contain ICS similar to that one used in the tested assay.

The data were statistically processed with Microsoft Excel. GraphPad Prism programme was used for graph plotting.

RESULTS AND DISCUSSION

Oligonucleotide design. Alignment and comparative assessment of nucleotides sequences of CSFV strains of different subgenotypes showed that, as expected, 5'-NTR was the most conservative genome region. Forward and reverse LNA-containing primers amplifying 120 nucleotides fragment were selected for this region (Fig. 1).

TaqMan probes for the target fragment of CSFV genome and for ICS fragment were labelled by fluorophores: 6-FAM (6-carboxyfluorescein) and Cy5.5 (cyanine-5.5), respectively.

Optimization of real-time RT-PCR conditions. Thermal-temporal profile and quantitative composition of the PCR mix were determined during the optimization.

The PCR mix for one reaction contained 1X OneTube RT-PCRmix, 0.4 pmol of forward primer and 0.4 pmol of reverse primer, 0.2 pmol of TaqMan-probe specific for the CSFV fragment; 0.1 pmol of forward primer and 0.1 pmol of reverse primer and TaqMan probe specific for ICS fragment, 1X TM-MMLV revertase, 10.0 µL of RNA matrix. Nuclease-free water was added to make the final reaction mix volume of 25.0 µL.

The PCR procedure included reverse transcription at 50 °C for 25 minutes and double-cycling amplification: general denaturation to inactivate revertase and activate DNA polymerase with a "hot-start" at 95 °C for 10 minutes, the first 10 cycles without fluorescence detection (denaturation at 95 °C for 10 seconds, primer annealing at 60 °C for 40 seconds, elongation at 72 °C for 10 seconds), then 35 cycles with fluorescence detection on the Green and Crimson channels (denaturation at 95 °C for 10 seconds, primer annealing at 55 °C for 40 seconds, elongation at 72 °C for 10 seconds).

ICS selection and optimization. MS2 bacteriophage is used as ICS for diagnosis of diseases caused by RNA viruses (hepatitis C, acquired human immunodeficiency syndrome, etc.). The phage of the *Leviviridae* family is a small icosahedral virion, pathogenic for *Escherichia coli*. The genome is made of single-stranded RNA of 3,569 nucleotides in length [20]. MS2 was chosen as ICS owing to its RNA genome and safety for humans, animals, and plants [21].

Intact ST cell culture, 10% spleen suspension samples collected from pigs infected with epizootic CSFV strain and from CSF-free pigs, culture CSFV 719 strain and NCS with different MS2 bacteriophage contents in the sample were tested to determine sufficient ICS concentration for adding to RNA extraction system (Table 1).

As a result, the optimal ICS amount was 3.2×10^3 PFU of MS2 bacteriophage per 100 µL of the sample. MS2 suspension with recommended titer was tested by storing at a temperature of +4 °C, and was found to have a stable threshold amplification cycle (Ct) with changes of ± 2 Ct when it was used for real-time RT-PCR after six-months storage. Ct value remained stable during five freezing (–20 °C) / thawing (room temperature) cycles.

Assessment of accuracy, repeatability and reproducibility. The developed assay under validation was shown to have 100% accuracy, repeatability and reproducibility during tests since known CSFV 719 strain-positive sample was found positive during 6 repeats in 3 parallel tests performed by one operator within one day and 3 parallel tests performed by two operators during three days (Table 2).

However, according to the graph (Fig. 2), Ct value increased by 3.09 ± 0.81 on day 3 of testing of the same sample as compared to that one on day 2 of testing by two operators that could be accounted for multiple freezing/thawing of the virus-containing material.

Assessment of analytical sensitivity. Mean minimum CSFV titers detectable by the real-time RT-PCR assay in 0.23 lg $CCID_{50}/cm^3$ were determined for CSFV 719, 684 and Shimen strains.

According to Table 3, comparative analysis of parallel tests of the virus strains using different real-time RT-PCR-based test systems showed that for CSFV 684 strain the detection limit of the developed assay was 1.2 lg higher than detection limits of test systems No. x1 and x2, and for reference Shimen strain the detection limit of the developed assay was 2.0 lg higher than that one of the test system No. x2. CSFV 719 strain-containing sample with a titer of 7.5 lg $CCID_{50}/cm^3$ was tested with the developed assay with positive results, while inconclusive results were obtained when the same sample was subjected to parallel tests using test systems No. x1 and x2.

A graph of correlation was plotted based on obtained Ct values ($n = 3$) and 10-fold dilutions of CSFV 719 strain (Fig. 3).

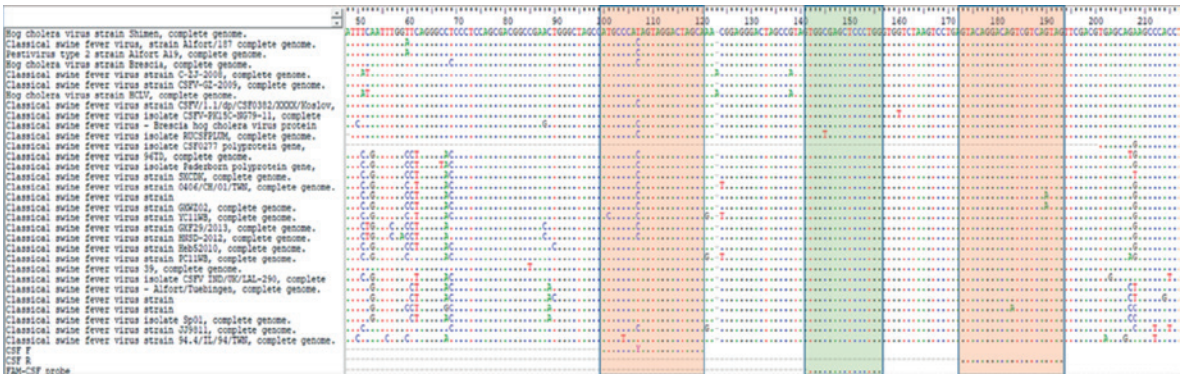

Fig. 1. Alignment of CSFV genome 5'-untranslated region (5'-NTR) sequences obtained from the GenBank (forward and reverse primer annealing sites are given in orange, TaqMan probe hybridization region is given in green)

Table 1
Results of internal control sample (ICS) titration with real-time RT-PCR using samples of different types

Sample	ICS titer (PFU/reaction)	Ct/Green	Ct/Crimson
NCS	10^5	–	20.22
Intact ST cell culture		–	26.02
10% spleen suspension from CSF-free pig		–	–
10% spleen suspension from CSFV infected pig		14.28	–
CSFV 719 strain-containing suspension		6.15	–
NCS	3.2×10^5	–	19.85
Intact ST cell culture		–	25.67
10% spleen suspension from CSF-free pig		–	22.80
10% spleen suspension from CSFV infected pig		13.62	23.22
CSFV 719 strain-containing suspension		5.66	–
NCS	10^6	–	14.11
Intact ST cell culture		–	15.93
10% spleen suspension from CSF-free pig		–	17.90
10% spleen suspension from CSFV infected pig		15.12	14.73
CSFV 719 strain-containing suspension		5.95	26.07
NCS	3.2×10^6	–	11.70
Intact ST cell culture		–	13.21
10% spleen suspension from CSF-free pig		–	15.21
10% spleen suspension from CSFV infected pig		12.10	10.48
CSFV 719 strain-containing suspension		5.56	24.25
NCS	10^7	–	12.34
Intact ST cell culture		–	17.82
10% spleen suspension from CSF-free pig		–	11.93
10% spleen suspension from CSFV infected pig		14.43	–
CSFV 719 strain-containing suspension		8.37	–

"–" – negative result; PFU – plaque-forming unit; Ct/Green – cycle threshold value for CSFV genome detection; Ct/Crimson – cycle threshold value for ICS detection.

The high statistical parameters calculated using correlation analysis were as follows: reaction efficiency E = 105%, adequacy coefficient $R^2 = 0.9928$ and significance criterion p value < 0.0001, that were indicative of the prospects of this real-time RT-PCR assay use for further development of quantitative PCR with reference samples.

Assessment of analytical specificity. Samples containing heterologous virus and bacteria genomes (ASFV, Aujeszky's disease virus, PRRSV, erysipelas agent and BVDV) were tested with the real-time RT-PCR assay with negative results and samples containing CSFV strains were tested CSFV RNA-positive with the real-time RT-PCR assay, so analytical specificity of the real-time RT-PCR assay was 100% (Table 4).

ICS fragment amplification was found to be inhibited at high Ct value on the Green channel (CSFV genome) that could be accounted for consumption of the reaction mix components (deoxyribonucleotide triphosphates, DNA polymerase, etc.) for the target PCR product synthesis with a large number of the matrix copies [20]. This should be taken into account when interpreting the results.

Determination of diagnostic sensitivity and specificity. All 27 true positive samples containing CSFV or collected from the pigs experimentally infected with epizootic CSFV strain as well as samples of the feed contaminated by infected animals were tested positive for CSFV genome with the real-time RT-PCR assay. Similarly, all 27 true negative samples were tested negative for CSFV RNA with the real-tine RT-PCR assay (Fig. 4).

Thus, the tests showed that diagnostic sensitivity and specificity of the assay were as high as possible and equaled to 100%. At the same time, the real-time RT-PCR-based test system No. x2 did not detect a positive blood sample collected from pigs experimentally infected with epizootic CSFV strain, as well as a positive feed sample collected in the animal facility where the pigs experimentally

Table 2
Developed real-time RT-PCR assay accuracy, repeatability and reproducibility values

Accuracy			
Sample	Ct/Green	(mean value ± SD)	Ct/Crimson
CSFV 719 strain	5.31	5.32 ± 0.148	–
CSFV 719 strain	5.22		27.29
CSFV 719 strain	5.30		26.01
CSFV 719 strain	5.52		27.27
CSFV 719 strain	5.33		26.67
CSFV 719 strain	5.28		29.37
Repeatability			
Procedure	Ct/Green	(mean value ± SD)	Ct/Crimson
First measurement	5.38	5.64 ± 0.237	28.63
First measurement	5.74		26.85
First measurement	5.71		22.68
First measurement	5.61		23.78
First measurement	5.59		23.87
First measurement	5.86		24.41
Second measurement	5.63	5.44 ± 0.493	23.14
Second measurement	5.37		23.95
Second measurement	5.02		23.62
Second measurement	5.11		23.11
Second measurement	5.96		23.01
Second measurement	5.56		23.70
Third measurement	5.59	5.60 ± 0.095	28.84
Third measurement	5.60		30.30
Third measurement	5.58		28.72
Third measurement	5.74		30.26
Third measurement	5.57		28.06
Third measurement	5.57		28.87

Reproducibility (Ct/Green)					
Day 1		Day 2		Day 3	
first operator	second operator	first operator	second operator	first operator	second operator
5.52	5.07	5.67	6.51	7.83	8.95
5.27	5.10	5.61	4.70	8.01	9.19
5.46	5.82	5.65	4.55	6.71	9.17
5.42	5.98	5.64	5.14	8.28	8.98
5.38	5.33	5.73	5.44	8.02	9.30
5.46	5.06	5.78	5.49	9.21	9.34
5.4183 ± 0.125	5.3933 ± 0.591	5.68 ± 0.0917	5.305 ± 1.019	8.01 ± 1.166	9.155 ± 0.233

"–" – negative result; Ct/Green – cycle threshold value for CSFV fragment; Ct/Crimson – cycle threshold value for ICS fragment.

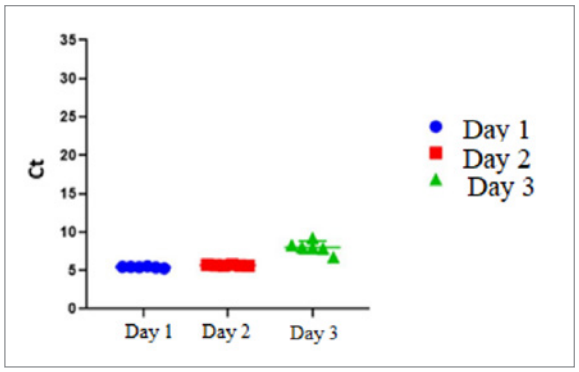

Fig. 2. Distribution of Ct values for known positive sample in different days of testing when the real-time RT-PCR assay was assessed for its reproducibility

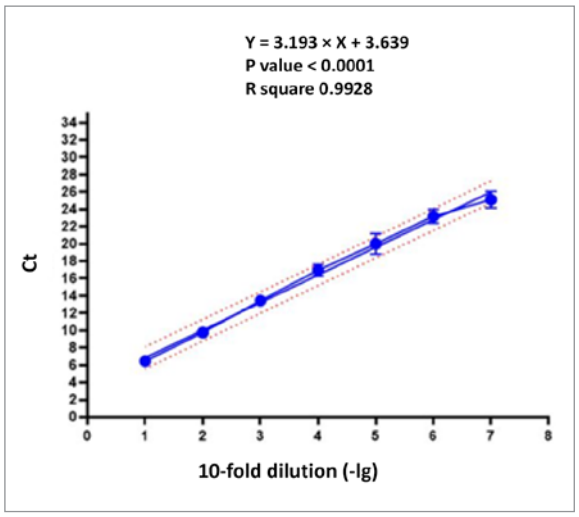

Fig. 3. Graph of linear correlation of CSFV 719 strain dilutions with Ct-values

infected with the same strain were kept, test-system No. x1 also did not detect the said positive blood sample that was indicative of their lower diagnostic sensitivity (92.6% for test system No. x2 and 96.3% for test system No. x1) than that one of the developed assay under validation.

Analysis and interpretation of the results. The recommended parameters of PCR assay for the Green and Crimson channels were identical: dynamic baseline setting, slope correction, emission reduction by 10%, linear scale, and threshold value of 0.05. The results were interpreted based on the presence or absence the standard curve intersection with the set threshold line that corresponds to Ct value presence or absence in relative line of the results table (Fig. 5).

Also, 2-fold dilution of the RNA extracted from CSFV 719 strain suspension with final titer of 7.5 lg $CCID_{50}/cm^3$ was tested to establish the PCR assay parameters and to determine maximum Ct value at which the sample could be interpreted as "positive" (Table 5).

The obtained data showed that the maximum Ct value was 27.83 that was equivalent to CSFV 719 strain titer of 0.23 lg $CCID_{50}/cm^3$.

The result was considered reliable when the correct results were obtained for positive and negative controls.

The sample was considered positive for CSFV genome when Ct value on Green channel did not exceed 28. In this

Table 3
Real-time RT-PCR results for 10-fold dilutions of various epizootic CSFV strains

Virus dilution	Shimen strain (titer – 4.0 lg $CCID_{50}/cm^3$)	719 strain (titer – 7.5 lg $CCID_{50}/cm^3$)	684 strain (titer – 6.2 lg $CCID_{50}/cm^3$)
	Ct/Green	Ct/Green	Ct/Green
Non-diluted	11.88 (pos)	5.41 (pos)	6.79 (pos)
$1:10^1$	16.18 (pos)	9.75 (pos)	9.89 (pos)
$1:10^2$	20.52 (pos)	12.53 (pos)	13.69 (pos)
$1:10^3$	22.64 (pos)	16.68 (pos)	16.97 (pos)
$1:10^4$	27.06 (pos)	18.79 (pos)	20.24 (pos)
$1:10^5$	–	20.60 (pos)	23.45 (pos)
$1:10^6$	–	21.57 (pos)	26.30 (pos)
$1:10^7$	–	24.71 (pos)	–
$1:10^8$	–	–	–
Test system No. x1			
Ct/Yellow (result)			
Non-diluted	12.89 (pos)	6.44 (pos)	8.27 (pos)
$1:10^1$	16.77 (pos)	8.33 (pos)	12.89 (pos)
$1:10^2$	18.67 (pos)	13.56 (pos)	15.08 (pos)
$1:10^3$	21.96 (pos)	17.67 (pos)	17.36 (pos)
$1:10^4$	25.78 (pos)	18.82 (pos)	22.85 (pos)
$1:10^5$	–	24.01 (pos)	26.57 (inconcl)
$1:10^6$	–	22.79 (pos)	–
$1:10^7$	–	28.19 (inconcl)	–
$1:10^8$	–	–	–
Test system No. x2			
Ct/Yellow (result)			
Non-diluted	19.07 (pos)	10.60 (pos)	9.37 (pos)
$1:10^1$	22.42 (pos)	13.34 (pos)	12.69 (pos)
$1:10^2$	25.28 (pos)	16.46 (pos)	16.13 (pos)
$1:10^3$	–	17.67 (pos)	19.19 (pos)
$1:10^4$	–	20.55 (pos)	22.22 (pos)
$1:10^5$	–	22.55 (pos)	24.68 (pos)
$1:10^6$	–	25.44 (pos)	–
$1:10^7$	–	28.02 (inconcl.)	–
$1:10^8$	–	–	–

"–" – negative result; pos – positive result; inconcl – inconclusive result;
Ct/Green – cycle threshold value for CSFV fragment; Ct/Yellow – cycle threshold value for CSFV fragment obtained when real-time PCR-based test systems No. x1 and x2 were used in accordance with their manufactures' instructions.

Table 4
Assessment of analytical specificity of the real-time RT-PCR assay when the assay was used for CSFV genome detection (*n* = 2)

Sample	Ct/Green	Ct/Crimson	Result of test for CSFV genome
Field *Erysipelothrix rhusiopathiae* isolate	–	19.08	negative
PRRSV Irkutsky 2007 strain	–	13.03	negative
Aujeszky's disease virus VK-DEP strain	–	13.73	negative
ASFV Mozambique-78 strain	–	15.39	negative
BVDV Chelyabinsk 2021 strain	–	15.31	negative
Reference CSFV Shimen strain	18.64	15.39	positive
CSFV Amur 19-10/WB-12555 strain	12.19	16.88	positive
CSFV Tigrovoe 16/WB-634 strain	17.26	18.02	positive
CSFV 275 strain	11.27	–	positive
CSFV 719 strain	6.56	–	positive
CSFV 843 strain	6.27	–	positive
CSFV 917 strain	6.24	–	positive
CSFV 918 strain	21.8	16.7	positive
CSFV 920 strain	21.16	14.88	positive
CSFV 926 strain	14.12	16.07	positive
CSFV 927 strain	13.61	17.91	positive
CSFV 930 strain	27.25	14.61	positive
CSFV 368 strain	10.01	19.04	positive
CSFV 589 strain	11.81	19.81	positive
CSFV 684 strain	6.79	17.15	positive
CSFV 870 strain	9.64	18.45	positive
CSFV 924 strain	13.26	18.89	positive
CSFV 925 strain	13.93	17.04	positive
CSFV 929 strain	15.28	19.12	positive
NCS	–	15.17	negative
PCS (vaccine SK strain of CSFV)	19.57	18.76	positive

"–" – negative result; Ct/Green – cycle threshold value for CSFV fragment; Ct/Crimson – cycle threshold value for ICS fragment.

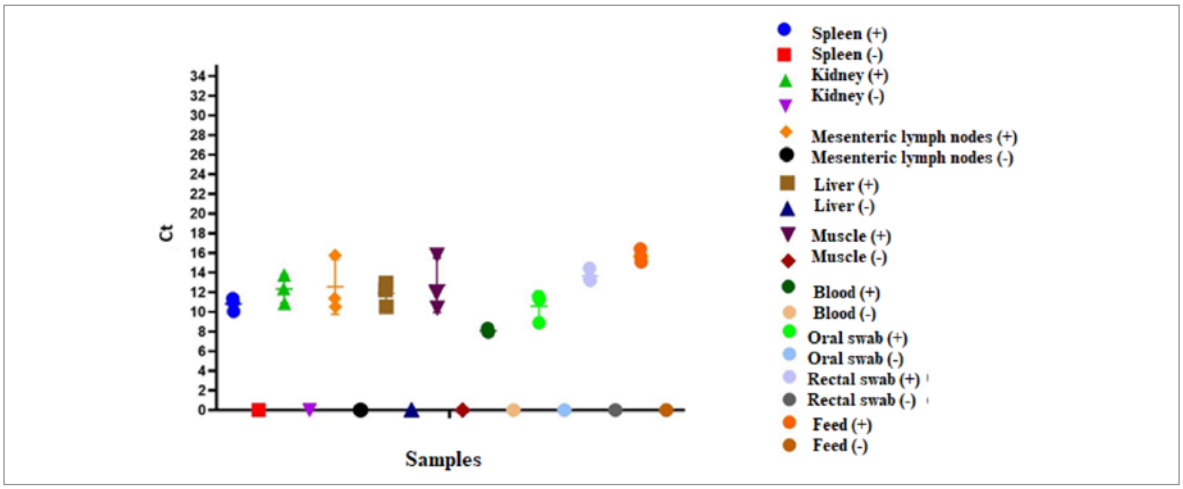

Fig. 4. Kinetics of Ct values for the panel of samples selected for determination of diagnostic sensitivity and specificity (samples – 10% suspensions of the indicated organs; "+" – true positive sample; "–" – true negative sample; l/n – lymph nodes; porcine raw meats and products were used as negative muscle samples)

Table 5
Real-time RT-PCR results for two-fold dilutions of CSFV RNA

CSFV RNA dilution	Ct/Green
non-diluted	6.04
$1:2^{1}$	6.86
$1:2^{2}$	7.55
$1:2^{3}$	8.75
$1:2^{4}$	12.02
$1:2^{5}$	11.75
$1:2^{6}$	12.06
$1:2^{7}$	12.90
$1:2^{8}$	13.42
$1:2^{9}$	14.61
$1:2^{10}$	15.61
$1:2^{11}$	16.32
$1:2^{12}$	17.19
$1:2^{13}$	18.21
$1:2^{14}$	19.27
$1:2^{15}$	20.14
$1:2^{16}$	21.17
$1:2^{17}$	22.08
$1:2^{18}$	22.98
$1:2^{19}$	24.06
$1:2^{20}$	24.74
$1:2^{21}$	24.22
$1:2^{22}$	26.13
$1:2^{23}$	26.18
$1:2^{24}$	27.83
$1:^{22}5$	27.01
$1:2^{26}$	–
$1:2^{27}$	–
$1:2^{28}$	–
$1:2^{29}$	–
$1:2^{30}$	–

CSFV RNA was extracted from CSFV 719 strain-containing suspension; titer 7.5 lg $CCID_{50}/cm^3$, "–" – negative result.

case, the result was valid regardless of the values on Crimson channel.

A result for the presence of the CSFV genome was interpreted as negative when there was no Ct value on Green channel, but Ct value on Crimson channel did not exceed 31.

If Ct value on Green channel exceeded 28, but on Crimson channel was less than 31 the PCR result was considered inconclusive.

When there were no Ct values on Green and Crimson channels, as well as when Ct value on Crimson channel exceeded 31, the PCR result was considered invalid.

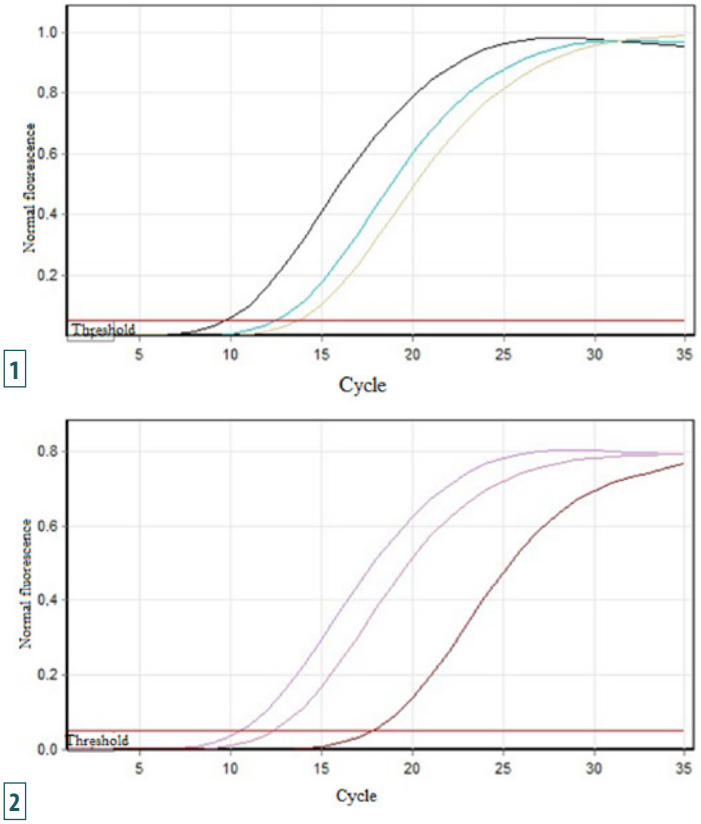

Fig. 5. Fluorescence curves: 1 – for Green channel (CSFV fragment); 2 – for Crimson channel (ICS fragment)

In case of inconclusive or invalid results the sample was to be retested starting from the RNA extraction stage in order to confirm absence or presence of CSFV genome in the sample.

CONCLUSION

The developed assay has high validation characteristics: 100% accuracy, repeatability, reproducibility, analytical specificity, diagnostic sensitivity and specificity, detection limit of 0.23 lg $CCID_{50}/cm^3$. Such characteristics make the developed real-time PCR-RV assay competitive on the domestic market of diagnostic tools and suitable for use for large-scale monitoring of CSF situation in Russia.

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Clinical signs and post-mortem lesions caused by clostridial enterotoxemia in rabbits

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ABSTRACT

Enterotoxemia, accompanied by diarrhea and bloating, is still a matter of pressing concern to the rabbit farming. *Clostridia* bacteria are often isolated from the internal organs of rabbits that have died of an anaerobic infection. Clostridial infection, manifested in various forms, is a major problem for veterinarians. The following drivers contribute to the emergence of the infectious disease: malnutrition (insufficient fiber intake); non-compliance with hygiene requirements for animal handling; unsustainable use of antibacterial drugs; gastrointestinal congestion. All these drivers can disrupt healthy caecum microflora due to changes in the gastrointestinal environment. Low-fiber diets result in slow cecum motility, thus, delaying transit of the intestinal contents and eventually changing the microflora. Use of antibiotics together with stress make *Clostridia* accumulate in the gastrointestinal tract, at the same time, reducing the number of microorganisms of other groups. The first signs of toxicoinfection are observed when rabbit kits are weaned from does. Clinical manifestation begins with bloating, weakness, inappetence, which ultimately lead to death. Observations have shown that the risk group includes rabbit kits weaned from the 35–77-day old does. Mortality was less reported in breeding stock and among replacement young animals. Autopsy revealed signs of enterotoxemia: serous-catarrhal gastritis, serous-hemorrhagic lymphonodulitis, degenerated kidneys, liver and heart muscle; passive congestion of lungs and pulmonary edema. Microbiological diagnosis revealed *Clostridium histolyticum* and *Clostridium perfringens* species known for their pronounced toxigenic profile, most often bacteria were found in the stomach, intestines and heart.

Keywords: enterotoxemia, *Clostridium*, rabbits, mortality, toxigenicity

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Клинические признаки и патолого-анатомические изменения при клостридиозной энтеротоксемии кроликов

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РЕЗЮМЕ

Проблема энтеротоксемии, сопровождающейся поносами и вздутием живота, продолжает оставаться достаточно острой в кролиководческой практике. Зачастую из внутренних органов кроликов, павших от анаэробной инфекции, выделяют микроорганизмы рода *Clostridium*. Клостридиозная инфекция в разных формах своего проявления является актуальной проблемой для ветеринарных врачей. Причинами возникновения данной инфекционной болезни являются: нарушения в кормлении (пониженное содержание клетчатки); несоблюдение зоогигиенических требований к содержанию животных; нерациональное использование антибактериальных препаратов; застой в желудочно-кишечном тракте. Все это может привести к нарушению баланса сложной микрофлоры слепой кишки вследствие изменения показателей среды желудочно-кишечного тракта. Диеты с низким содержанием клетчатки вызывают гипомоторику слепой кишки, продлевая задержку в ней содержимого и в конечном счете вызывая изменения ее микрофлоры. Применение антибиотиков и стрессы способствуют накоплению клостридий в желудочно-кишечном тракте с одновременным снижением других групп микроорганизмов. Первые признаки токсикоинфекции наблюдаются при отъеме крольчат от самок. Манифестация клинических признаков начинается со вздутия живота, вялости, отсутствия аппетита, что в итоге приводит к летальному исходу. Как показали исследования, в группу риска входили крольчата после отъема от самок в возрасте 35–77 сут. Реже падеж наблюдали среди маточного поголовья и в группе ремонтного молодняка. При вскрытии установлены признаки энтеротоксемии: серозно-катаральный гастрит, серозно-геморрагический лимфонодулит, дистрофия почек, печени и сердечной мышцы, легкие в состоянии застойной гиперемии и отека. При микробиологической диагностике выявили наличие микроорганизмов видов *Clostridium histolyticum* и *Clostridium perfringens* с выраженными токсигенными свойствами, наиболее часто бактерии обнаруживали в желудке, кишечнике и сердце.

Ключевые слова: энтеротоксемия, клостридии, кролики, падеж, токсигенность

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INTRODUCTION

A growing interest in healthy foods stimulates rabbit meat consumption in Russia, therefore, studying infectious diseases of rabbits is an urgent scientific task. Rabbit meat production is a profitable business due to a rapid herd replacement[1]. However rabbit raising is associated with some specific problems that result from diseases and subsequent mortality.

In the rabbit farming, veterinarians often have to encounter enteritis symptoms accompanied by diarrhea. As a result, enterotoxemia, septic processes leading to the animal death can develop. Enterotoxemia is always caused by various microorganisms [1].

Enterotoxemia in rabbits is very often caused by the enterotoxigenic *Clostridium spiroforme* strain, when the normal microbiome is disturbed. Most often, animals got diseased at the age of 3–6 weeks after weaning. Gut microflora of young rabbits is characterized by either an insufficient number or absence of its normal representatives and by a high pH in the stomach which cause rapid multiplication of *C. spiroforme* [2]. The highest mortality rate is recorded in baby rabbits during this period. Clinical signs of acute *C. spiroforme* caused enterotoxemia include: inappetence; lethargy; brown watery diarrhea with blood and mucus resulting in stained perineum and hind limbs. As the disease progresses, body temperature decreases, death occurs after 24–48 hours [3]. Autopsy reveals petechial and ecchymotic hemorrhages on the cecum serous membrane. The appendix and the proximal colon may be affected, where hemorrhages and/or mucus are observed [4].

Clostridium bacteria occur due to a decreased immunological resistance of the body, gastrointestinal diseases and metabolic disorders [5]. Pathogenic mechanisms of gastrointestinal diseases in rabbits are launched when normal peristalsis is impaired and normal peristalsis can be ensured only by large amounts of indigestible fiber [6]. When intestinal motility is impaired, the liquid is absorbed from the stomach, further condensing its contents. Condensed food causes discomfort, which further contributes to anorexia and exacerbates reduced motility of the gastrointestinal tract. Insufficient fiber intake resulting either from malnutrition or from conditions that cause anorexia is the main cause of gastrointestinal congestion. Fiber improves the cecum motility either through increasing the volume of the contents, or directly. High fiber diet stimulates generation of special volatile fatty acids in the caecum, which ensure proper work of gastrointestinal tract [7].

Slow motility changes pH of the cecum contents, increasing the level of *Clostridium* spp. and coliform species such as *Escherichia coli*, at the same time reducing the population of good gut microorganisms. Small amounts of these potentially pathogenic bacteria are usually observed in the caecum [8]. The cecum functions as a fermentation chamber and contains a complex microbiome, including anaerobic microorganisms such as *Bacteroides*, large metachromatically stained bacteria, gram-negative oval and spindle-shaped rods, yeast, several non-pathogenic species of protozoa and amoebas, and many still unexplored bacterial species [9, 10]. This microbial population ensures processing of fiber into easily digestible nutrients, which then re-enter the body due to cecotroph consumption [11, 12].

Bacteroidetes and *Firmicutes* species are dominant microorganisms of the rabbit jejunum. The former are mainly involved in the metabolism of carbohydrates, steroids and other nutrients, supporting the morphology and physiological function of the intestinal tract and microflora balance. *Firmicutes* play an important role in carbohydrate metabolism [13]. Among *Firmicutes* representatives, clostridia predominate in the intestinal microbiota. They are involved in the process of nutrient absorption and synthesis of short-chain fatty acids [14]. High levels of these bacteria can cause a number of pathological changes: from abdominal bloating to death from enterotoxemia. Gas and toxin generation is accompanied by pain; because of stress the rabbits gradually lose appetite till it is lost completely, hypomobility is observed.

An increased level of carbohydrates creates an environment which benefits the growth of such pathogens as *E. coli* and various *Clostridium* species [15]. Glucose is a product resulting from a breakdown of complex carbohydrates, which is required by *Clostridium* bacteria to produce iota toxin. Thus, diarrhea and enterotoxemia in domestic rabbits are often caused by microflora imbalance, commonly called dysbacteriosis [16].

Concomitant factors that contribute to the growth of pathogenic bacteria are also important: i.e. stress, inappropriate foods, use of antibiotics, and a genetic susceptibility to intestinal disorders [17, 18]. The development of acidosis and increased acidity in the stomach make

[1] Marketing research: Rabbit meat market for 2018–2022 http://ikc.belapk.ru/upload/iblock/976/976166bcbfb2b60b2440e64aa60f11aa.pdf (in Russ.)

the *Clostridium* biomass grow, accumulate and develop virulence. The bacteria can also be activated by a number of stress factors (such as abrupt changes in feeding associated with a decrease in carbohydrate levels, an increase in protein amounts; regrouping, transportation of animals, etc.) [19]. The active bacterial growth is accompanied by release of large amounts of exotoxins, which have local effects on the intestinal mucosa or are absorbed into the blood, thus causing more severe systemic damage to the body, and act as the key factor behind *Clostridia* pathogenicity. Some low-toxin-producing bacteria (as *C. chauvoei*, *C. septicum*) have flagella that contribute to virulence of pathogenic bacteria and also provide hemagglutination [20].

The *Clostridium* group includes several pathogenic species that cause intestinal signs, as well as neurotoxic or histotoxic infections. Intestinal infections in animals are mostly caused by *C. perfringens*, *C. difficile*, *C. histolyticum* and *C. septicum*: *C. perfringens* causes such intestinal signs as enterocolitis, enterotoxemia, and gastritis. According to A. V. Supova et al., the most common anaerobes included *C. perfringens*, *C. sporogenes*, *C. bifermentans*, *C. septicum*, *C. cadaveris*, *C. tertium*, *C. difficile*, *C. novyi*, *C. baratii* [1].

With clostridiosis, intrauterine or early postnatal infection is possible. Infection may be spread by a contact with milk, colostrum, litter, environmental objects contaminated with feces of adult animals, feed, soil, water [2, 7]. In case of intrauterine infection, the pathogen penetrates into the uterus with blood flow, which leads to intrauterine infection of young animals, abortions, metritis and endometritis. Members of *Clostridium* genus cause mastitis along with microorganisms of other families [21].

Clostridiosis on Siberian livestock farms mostly proceeds as a co-infection [22].

Given the segmentation of the rabbit breeding market and its status of a developing industry in Western Siberia, the problems faced by veterinarians are poorly understood and need to be considered in detail. In particular, the novelty of this research is that it is focused on studying enterotoxemia caused by *Clostridium* on a rabbit breeding farm in Western Siberia.

Therefore, the purpose of this work is to study etiology, epizootic data, clinical patterns and post-mortem lesions in clostridious enterotoxemia-infected rabbits on a breeding farm of Western Siberia.

MATERIALS AND METHODS

For the research purposes the following pathological materials were used (liver, spleen, stomach contents, intestinal contents, lungs, heart, kidneys); taken from dead or emergently killed sick rabbits of different age groups, i.e. from birth to 35 days old – 21 rabbits; 35–77 days old – 45 rabbits; replacement young rabbits from 35–71 days old – 14 rabbits; breeding stock from 72 days old – 9 rabbits.

The animals are bred on a closed-type rabbit breeding farm located in the south of Western Siberia, in cages equipped with automatic feeding and watering systems. The water supply is non-municipal. The water is supplied from a well with a water treatment system. Manure is removed daily by a scraper conveyor and is transported to a manure storage facility located at least 1 km away from the farm. The rabbit diet includes commercial balanced compound feeds. All mature rabbits (females, males, fattening, replacement rabbits) are kept in one house without "all in/all out" production principle. Disinfection is carried out only in the presence of animals, since there is no room for their regrouping during antiepidemic work. The livestock has been vaccinated against viral hemorrhagic disease and rabbit myxomatosis. In accordance with the plan of antiepizootic measures, preventive measures are taken against coccidiosis and helminthiasis. The farm is considered free from infectious diseases.

The animals were treated with oral antibiotics administered via drinking water: fluoroquinolone ("Enroflon 10%") 5–7 days – 1 g/L of water, sulfonamides ("Trimethoprim", "Zinaprim") 5–7 days – 1 g/L of water. Also, "Nitox 200" (tetracycline group) was administered intramuscularly once at a dose of 0.1 mL/kg of body weight. The breaks between taking medications were 7–10 days.

In 2023, *the bacterial strains were cultivated and identified* using classical bacteriological analysis. Cultivation and identification was performed in the research laboratory "Biochemical, molecular-genetic studies and selection of farm animals" of the Kuzbass State Agrarian University named after V. N. Poletskov.

The dead animals were delivered to the laboratory in a thermocontainer with cooling agents immediately after death (maximum within 30 minutes). Corresponding autopsy was performed, followed by examination of post-mortem lesions and diagnosis.

Nutrient media and cultivation conditions. Pieces of organs were inoculated into 5% meat-peptone broth (MPB) with glucose, incubated for 18–24 hours at 37 °C under anaerobic conditions. After incubation, primary cultivation was performed in Wilson and Blair Medium (incubation for 24–48 hours at 37 °C under anaerobic conditions). Blackening of the medium due to gas production, seen as cracks and bubbles in the dense medium, together with a very persistent and unpleasant odor of butyric acid shall be considered a positive result of *Clostridium* cultivation.

The following tests were performed to identify the genus of the recovered microorganisms: Gram staining, growth in MPB containing 6.5% NaCl and 20% bile (incubation for 24 hours at 37 °C under anaerobic conditions), hemolysis on 5% blood agar (incubation for 24 hours at 37 °C under anaerobic conditions), catalase test (with hydrogen peroxide), oxidase test (commercial OXITEST kit), indole test (Kovac's reagent).

Species were identified on differential diagnostic Gissa's media with arabinose, dulcite, fructose, galactose, glucose, inositol, inulin, lactose, maltose, mannitol, mannose, melesitose, melibiose, raffinose, rhamnose, ribose, salicin, sorbitol, sorbose, sucrose, trehalose and xylose (incubation for 24 hours at 37 °C under anaerobic conditions). After incubation, change in the media color together with gas production were used to assess the ability of the isolated cultures to ferment.

Toxigenic properties were determined by intraperitoneal injection of microbial suspension to 70 white mice in an amount of 0.5 mL at a dose of 500 million microbial cells grown on 5% blood agar (incubation for 24 hours at 37 °C under anaerobic conditions). Post-injection death of the animal was considered as a positive result.

All experiments in animals were conducted in strict compliance with interstate Guidelines for accommodation and care of animals (GOST 33216-2014 and GOST 33215-2014), adopted by the Interstate Council for

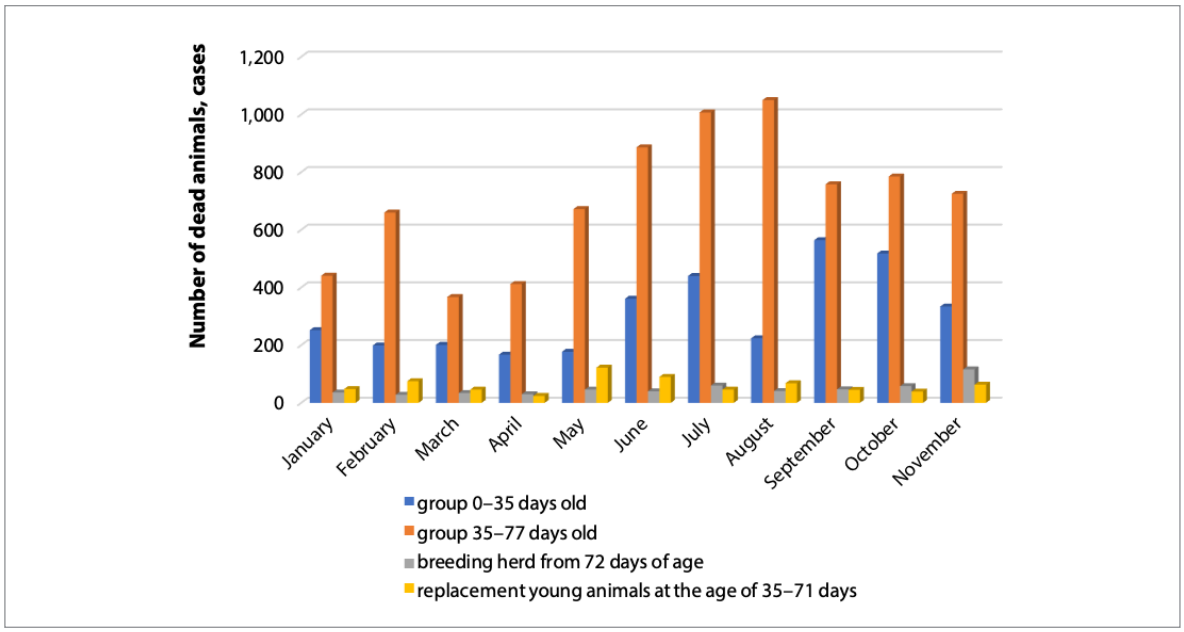


Fig. 1. The number of rabbits of different age groups that died in 2023

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.

To confirm death cases caused by *Clostridium* microorganisms, smears were prepared from the affected parenchymal organs of mice, using Gram staining. At the same time, in order to ensure more distinct staining of spores, dye solution on the microscope slide was heated over a burner flame to form vapors, which made it possible to identify *Clostridia*. Large rod-shaped gram-positive microorganisms in the smear without a visualized spore (*C. perfringens*) or with a terminal spore exceeding the diameter of the vegetative cell (*C. histolyticum*) was the evidence of *Clostridium* presence.

Statistical data processing. Percentage for the relative values of the isolated microorganisms was calculated by dividing the number of the isolated microorganisms from each organ by the total number of isolates, multiplied by 100%[2].

RESULTS AND DISCUSSION

Between January and November of 2023, mortality among rabbits (12,402 death cases) was reported on the farm in all sex and age groups. The situation was defined as mortality, if there were more than 5 dead animals in one group per one day, with typical clinical symptoms such as weakness, emaciation, ruffled fur, bloating, foul-smelling stool (if the number of death cases was less than 5, it was considered to be a regular death rate resulting from non-infectious courses: injuries, physiological characteristics, etc.). The mortality was reported in all fattening groups, less often in adult females and males from 1 to 3 years old (Fig. 1). Most death cases (1,050 animals, aged 35–77 days) were reported in August; peak mortality in the age group of 0–35 days was observed in September (564 rabbits); the maximum number of young replacement rabbits died in May (122 rabbits) and in the breeding herd in November (116 rabbits).

The following clinical symptoms were observed in animals of the age group starting from birth up to 35 days: bloating, foul-smelling stool, ruffled fur, emaciation, dehydration. Death occurred 1–2 days after detection of the signs. The greatest number of death cases was reported in the age group from 35 to 77 days – 7,763 animals; at the same time, the clinical signs were similar to those observed in the group of young animals under 35 days of age.

The clinical signs in does included stillbirths (20% of the offspring), lethargy, bloating, kits from such does were weak, poorly fed and died on days 3–5 after birth.

Post-mortem lesions were typical for general toxicosis:

– the dead rabbits looked emaciated, with clearly seen bone structures;

– bloated stomach;

– fur around the anal area and tail was contaminated with fecal material;

– dry subcutaneous tissue;

– pale and degenerated skeletal muscles;

– stomach mucous membrane is swollen, red, with a lot of mucus (catarrhal gastritis);

– mucous membrane of the small intestine is diffusely thickened with hemorrhages and large amounts of mucus;

– enlarged mesenteric lymph nodes, with sporadic redness or drainage from incision, with changes typical for acute serous hemorrhagic inflammation;

– spleen usually without visible changes;

– liver is slightly enlarged, flabby, unevenly colored, gray-yellowish with red spots;

– the kidneys are enlarged, slightly softened, of grayish-clay color, the border between the cortical and cerebral layers is unclear;

– enlarged heart resulting from expansion of cardiac cavities, mainly the right ones; flabby, the incision surface is grayish;

– passive congestion of lungs and pulmonary edema.

[2] Shorokhova I. S., Kislyak N. V., Mariev O. S. Statistical methods of analysis: a textbook. Yekaterinburg: Ural University Press; 2015. 300 p. https://elar.urfu.ru/bitstream/10995/36122/1/978-5-7996-1633-5_2015.pdf (in Russ.)

As bacteriological tests show, *Clostridia* grew mostly from pathological material sampled from animals at the age of 35–77 days, less often from those older than 72 days (Fig. 2). The main organs infected with *Clostridium* spp. were: stomach (12–89%), intestines (24–74%), heart (10–64%). Bacteria were also found in liver (5–17%), kidneys (15–21%) and lungs (3–46%).

Biochemical typing of the isolated *Clostridium* cultures revealed the following two species: *C. histolyticum* and *C. perfringens* (Fig. 3). *C. histolyticum* was isolated from pathological material taken from young animals under 35 days of age, i.e. in 43% of cases (9 rabbits); *C. perfringens* was isolated in 19% of cases (4 rabbits). The percentage of *C. histolyticum* isolated from samples taken from rabbits at the age of 35–77-days was 42% (19 rabbits), *C. perfringens* – 47% (21 rabbits). *C. histolyticum* was isolated in 43% of cases (6 rabbits), *C. perfringens* – in 29% of cases (4 rabbits) from the biological material taken from dead or emergently slaughtered replacement young animals of 35–71 days of age. *C. histolyticum* was found in 33% (3 rabbits) of the samples from rabbits of the breeding stock older than 72 days.

The number of dead laboratory animals accounted for 66. These are the animals in whose internal organs gram-positive large rods with rounded ends or large gram-positive rods with a subterminal spore were detected.

Analysis of the on-farm epizootic situation revealed the reasons behind the disease situation.

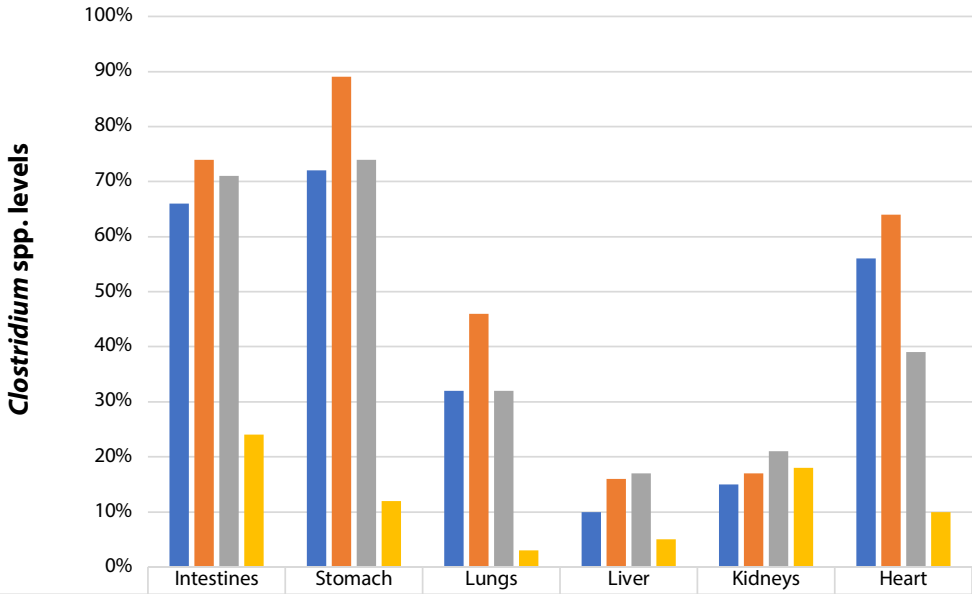
1. Non-compliance with the requirements for animal handling, namely: no isolation ensured for rabbits of different age groups; no conditions for disinfection when animals are moved to other premises. Unsatisfactory environment for animal keeping is one of the reasons for propagation and spread of various microorganisms, including *Clostridia*, which is confirmed by research results provided by other authors [2, 7, 17, 18]. Violation of veterinary and sanitary rules and rabbit keeping approaches have a negative impact on the epizootic situation on the farm. Prevention of bacterial diseases consists in the application of an integrated control system, which includes: monitoring; use of effective antibacterial products and their alternatives; disinfection quality control; HACCP principles in production. Until a new group of animals arrives, highly resistant clostridium spores spread in the facilities due to poor-quality sanitation [23, 24].

2. Use of antibiotics to control opportunistic microorganisms has resulted in selection and accumulation of toxigenic *Clostridium* strains. This fact is widely confirmed by L. N. Mazankova and S. G. Perlovskaya [25], whose studies demonstrate a key role of antibiotics in spread of clostridium due to death of endogenous microflora, without which *Clostridia* actively multiply and release toxins.

3. The group that was most susceptible to clostridiosis included weaned baby rabbits older than 35 days of age, because weaning is one of the stress factors that, combined with the rearrangement of animals and a switch to commercial feeds, undermines nonspecific protection. The fact is confirmed by other studies as well [26, 27] that show that rabbits aged between 3 and 7 weeks are highly susceptible to *Clostridia*-associated intestinal diseases.

CONCLUSION

Clostridiosis in rabbits on this farm is caused by two toxin-realising species: *C. histolyticum* and *C. perfringens*. The most susceptible group included weaned baby rabbits at the age of 35 to 71 days. At the same time, rabbits of this group demonstrated pronounced clinical signs and pathological and anatomical lesions typical for enterotoxemia: inflammation of the stomach and intestines, dystrophic



	Intestines	Stomach	Lungs	Liver	Kidneys	Heart
group up to 35 days old	66%	72%	32%	10%	15%	56%
group 35–77 days old	74%	89%	46%	16%	17%	64%
replacement young animals 35–71 days of age	71%	74%	32%	17%	21%	39%
breeding herd from 72 days of age	24%	12%	3%	5%	18%	10%

Fig. 2. Clostridium levels in pathological material from rabbits

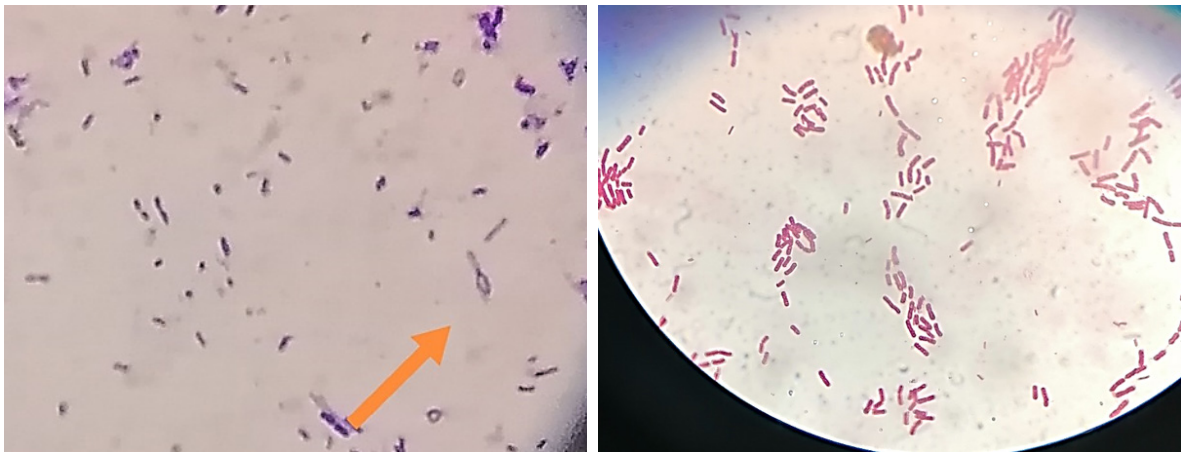


Fig. 3. C. histolyticum (left) and C. perfringens (right) in Gram-stained smears

changes in liver, kidneys and myocardium. Adult animals demonstrated less pronounced clinical signs which mainly included bloating, stillbirths and non-viable births. *Clostridium* was most often isolated from gastric, intestinal and cardiac tissue biopsies. *Clostridium* spp. was less isolated from lungs, liver and kidneys. The pathological and anatomical picture in clostridiosis is characteristic of general toxicosis: exhaustion, bloating, serous-catarrhal gastritis, enteritis, serous-hemorrhagic lymphodulitis of mesenteric lymph nodes, liver, myocardium and kidneys in a state of dystrophy, congestive hyperemia and edema in the lungs.

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Contribution: The authors made an equal contribution to the research: collected and analyzed the materials; specified goals and objectives and research methods; formulated scientifically-based conclusions; were involved in writing the article on the key research results.

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Effective measures to control eimeriosis in poultry in the Republic of Dagestan

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ABSTRACT
The most common disease of young poultry in commercial farms of the Russian Caspian region is eimeriosis. In most cases, after convalescence from coccidiosis caused by one of *Eimeria* species poultry remains susceptible to other species. This parasite has a very short life cycle and immense reproductive capacity that is why it can cause large-scale outbreaks of the disease in commercial poultry houses. To control avian eimeriosis, various coccidiostats are used in combination with probiotics and vitamins. Frequent and long-term use of the same drugs against this infection can potentially result in the emergence of resistant *Eimeria* populations. This suggests that this coccidiosis control requires rotation of eimeriocidal drugs. Studies on eimeriosis prevalence were performed in the laboratory of the Caspian Regional Research Veterinary Institute and in different poultry farms of the Republic of Dagestan. Swabs of the floor, litter, equipment, droppings, feedstuffs, cecum smears from dead poultry were used for testing. High infection rate with eimerias was established in floor-housed poultry in the plain and piedmont zones of the Republic (Khasavyurtovsky and Karabudakhkentsky raions), where the infection rates were 81.6 and 82.4%, respectively. In batter-cage system poultry farms of the mountain and mountain valley zones (Khunzakhsky and Gergebilsky raions) the infection rates were significantly lower – 61.2 and 58.0%, respectively. The comparative efficacy study of two eimeriocidal drugs showed that "Robenidine", used daily from the first day of life during the entire rearing period at a dose of 33 g per 1 ton of feedstuffs controls coccidiosis in poultry. At the same time, the survival rate of the experimental young poultry during the observation period was 98.0% compared with "Sarucoxum 12%" group (96.7%).

Keywords: broilers, eimeriosis, oocysts, eimerias, eimeriocidal drugs, "Robenidine", "Sarucoxum 12%", efficacy, infection rate, ceca, droppings

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Эффективные меры борьбы с эймериозами птиц в условиях Республики Дагестан

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РЕЗЮМЕ
Самым распространенным заболеванием молодняка на птицефабриках промышленного типа в Прикаспийском регионе России является эймериоз (кокцидиоз). В большинстве случаев птица, которая переболела кокцидиозом, вызванным одним видом эймерий, остается восприимчива и к другим видам возбудителя. У данного паразита очень короткий жизненный цикл и огромная репродуктивная способность, вследствие чего в птичниках промышленного типа случаются массовые вспышки заболевания. Для борьбы с эймериозами птиц применяют различные кокцидиоцидные препараты в сочетании с пробиотиками и витаминами. Частое и долгосрочное использование одних и тех же средств лечения данной инвазии приводит к возникновению устойчивых популяций эймерий. Это говорит о том, что при борьбе с этим паразитозом важно чередовать эймериоцидные препараты. Исследования по изучению распространения эймериоза проводили на базе лаборатории Прикаспийского зонального научно-исследовательского ветеринарного института и в птицеводческих хозяйствах Республики Дагестан различного типа. Материалом для исследований служили соскобы с пола, подстилки, инвентаря; помет; корма; мазки-отпечатки слепых отростков кишечника павшей птицы. Выявлена высокая зараженность эймериями птиц, выращиваемых в условиях напольного содержания в равнинной и предгорной зонах республики (Хасавюртовский и Карабудахкентский районы), где уровень инвазирования составил 81,6 и 82,4% соответственно. В птицеводческих хозяйствах горной зоны и зоны горных долин (Хунзахский и Гергебильский

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районы) при клеточном выращивании степень поражения птиц была значительно ниже – 61,2 и 58,0% соответственно. При сравнительном изучении эффективности двух эймериоцидных препаратов установлено, что «Робенидин» ежедневно с первого дня жизни в течение всего периода выращивания в дозе 33 г на 1 тонну корма санирует организм птицы от паразитов. При этом выживаемость подопытного молодняка птицы за период наблюдения составила 98,0% по сравнению с группой, где применяли «Сарукокс 12%» (96,7%).

Ключевые слова: цыплята-бройлеры, эймериоз, ооцисты, эймерии, эймериоцидные препараты, «Робенидин», «Сарукокс 12%», эффективность, интенсивность инвазии, слепые отростки кишечника, помет

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INTRODUCTION

The most common disease of young poultry in commercial farms of the Russian Caspian region is eimeriosis (coccidiosis) [1].

The scientists point out that eimeriosis mainly affects young poultry (10 day – 3-month old) and attribute this to underdeveloped immunity [2, 3, 4, 5, 6].

In most cases, after convalescence from coccidiosis caused by one of *Eimeria* species poultry remains susceptible to other species. This parasite has a very short life cycle and immense reproductive capacity that is why it can cause large-scale outbreaks of the disease in commercial poultry houses [6, 7, 8, 9].

Many authors describe the enormous damage caused to poultry farming by poultry deaths, reduced weight gain and meat production [10, 11, 12, 13].

To control avian eimeriosis, various coccidiostats are used in combination with probiotics and vitamins [14, 15, 16, 17].

The peak incidence in poultry is most often observed during warm and humid periods of the year (spring and late autumn).

The scientists note that 4–10 *Eimeria* species can infect poultry at the same time, which significantly complicates this disease control [6, 9].

In recent years, co-infections with eimerias and cryptosporidias, salmonellas and colibacterias have been frequently reported [18].

Frequent and long-term use of the same drugs against this infection can potentially result in the emergence of resistant *Eimeria* populations. This suggests that control of this coccidiosis requires rotation of eimeriocidal drugs [19, 20, 21].

The aim of the research was to study eimeriosis prevalence in poultry farms of the Republic of Dagestan and the efficacy of "Robenidine" and "Sarucoxum 12%" by comparison.

MATERIALS AND METHODS

Commercial drugs "Robenidine" and "Sarucoxum 12%" were tested.

"Robenidine" has a coccidiostatic effect against the main species of avian coccidia (*Eimeria necatrix*, *Eimeria tenella*, *Eimeria acervulina*, *Eimeria brunetti*, *Eimeria maxima*, *Eimeria mivati*), at the stage of first and second generation schizonts. "Robenidine" affects energy metabolism of coccidia cells and adversely affects the process of nuclear division which leads to the death of parasites.

"Sarucoxum 12%" contains salinomycin sodium, a polyether ionophore antibiotic. The drug disrupts the transport of sodium and potassium ions in the oocyst and leads to the death of coccidia at the stage of schizogony. It has an anticoccidial effect against all coccidia species of poultry and other livestock.

The experiments were performed in the laboratory of the Caspian Regional Research Veterinary Institute and commercial poultry farm AO "Poultry farm "Makhachkalinskaya", as well as in other poultry farms of the region.

Swabs of the floor, litter, equipment, droppings, feedstuffs, cecum smears from dead poultry were used for testing.

Laboratory tests to establish the diagnosis and *Eimeria* infection rate in poultry were carried out in accordance with GOST 25383-82 (ST SEV 2547-80) "Domestic animals. Methods of laboratory diagnostics of coccidiosis"[1].

To conduct an experiment in a commercial poultry farm of AO "Poultry farm Makhachkalinskaya", 3 groups of chicks were formed: 2 test groups and 1 control group (150 chicks per group).

Test group 1 broilers received "Robenidine" daily from the first day of life during the entire rearing period at a dose of 33 g per 1 ton of feedstuffs. The drug was removed from

[1] https://docs.cntd.ru/document/1200025474?ysclid=lwmb1ge0n7566471140 (in Russ.)

Table 1
Prevalence of eimerioses in poultry in the raions and different attitudinal zones of the Republic of Dagestan

Raions and zones	Tested for eimeriosis				
	No. of samples	subjected to post-mortem examination	subjected to microscopy	positive samples detected	%
Khasavyurtovsky (plain zone)	364	104	364	297	81.6
Karabudakhkentsky (piedmont zone)	256	84	256	211	82.4
Khunzakhsky (mountain zone)	178	34	178	109	61.2
Gergebilsky (intermontane valley zone)	188	32	188	109	58.0
TOTAL	986	254	986	726	73.6

the diet 5 days before slaughter. Test group 2 was treated with "Sarucoxum 12%" at a dose of 7 mg/kg, which is equivalent to 1 mL per 1 liter of drinking water given within 48 hours. Control chicks were not treated.

Poultry were observed during the entire period of the experiment. Treated chicks were tested for oocysts on days 16, 26, 36, 48.

The experiments on animals were carried out in accordance with GOST 33215-2014, adopted by the Interstate Council for Standardization, Metrology and Certification, as well as in accordance with the requirements of the Helsinki Declaration (2000) and Directive 2010/63/EU of the European Parliament and of the Council of the European Union dated 09/22/2010 on the protection of animals used for scientific purposes.

The intensive efficacy (IE) of the drugs was determined by coproscopy for *Eimeria* oocysts in ceca and droppings.

The effect of the drugs on poultry performance was evaluated by clinical signs manifested; oocyst index and mortality rates caused by eimeriosis, as well as weight gains in control and test groups.

A McMaster or VIGIS counting chamber was used to count oocysts in 1 g of droppings.

The results were statistically processed using Biometrics software.

RESULTS AND DISCUSSION

The eimeriosis situation in different altitudinal zones of the region was studied at the poultry farms and small-scale farms practicing floor and battery cage system rearing, located in Khasavyurtovsky (plain zone), Karabudakhkentsky (piedmont zone), Khunzakhsky (mountain zone) and Gergebilsky (intermontane valley zone) raions of the Republic.

986 carcasses of dead and killed poultry were examined, including 254 birds subjected to post-mortem examination, and 986 birds subjected to microscopy. There were 726 positive results which is 73.6% (Table 1).

In the plain and piedmont zones of the Republic (Khasavyurtovsky and Karabudakhkentsy raions), a high *Eimeria* infection rate in floor-housed poultry was revealed. The prevalence was 81.6 and 82.4%, respectively.

A completely different picture is observed in the cage-housed poultry at the farms of the mountain zone and intermontane valley zone (Khunzakhsky and Gergebilsky raions) infection, where the prevalence of infection was 61.2 and 58.0%, respectively.

The laboratory tests revealed eimerias in ceca, duodena and droppings of broilers (Table 2).

According to the literature data the morphology of the eimerias detected in pathological samples (droppings, ceca and duodena) was consistent with *Eimeria tenella*, *Eimeria maxima*, *Eimeria mitis*, *Eimeria acervulina* species.

To control avian eimeriosis successfully, it is necessary to conduct constant research and development of modern highly effective eimeriocidal drugs, therefore, one of the stages of the study was a comparative efficacy study of "Robenidine" and "Sarucoxum 12%" against spontaneous eimeriosis in broilers in a commercial poultry farm (battery cage system) of AO "Poultry farm "Makhachkalinskaya" (Table 3 and 4).

Table 2
The results of testing of broiler intestines and droppings for eimerias

Raions and zones	No. of samples	*Eimeria* oocysts detected (No. seen in a single field of view)		
		in cecum	in droppings	in duodenum
Khasavyurtovsky (plain zone)	104	54–56	8–10	7–9
Karabudakhkentsky (piedmont zone)	84	24–26	4–5	4–6
Khunzakhsky (mountain zone)	34	11–12	2–3	1–2
Gergebilsky (intermontane valley zone)	32	6–8	2–3	1–2
TOTAL	254	–	–	–

Table 3
Design of testing in cage-housed ROSS 308 broilers

Groups	Drug	Number of chicks	Dose and course of treatment
Group 1	"Robenidine"	150	Daily from the first day of life during the entire rearing period at a dose of 33 g per 1 ton of feedstuffs. The drug was removed from the diet 5 days before slaughter
Group 2	"Sarucoxum 12%"	150	Given with water during 48 hours at a dose of 7 mg/kg, which is equivalent to 1 mL per 1 liter of drinking water
Control	–	150	–

Table 4
The efficacy of eimeriocidal drugs against spontaneous eimeriosis in broilers

Indicator	Control	Test groups	
		"Robenidine"	"Sarucoxum 12%"
Before treatment			
No. of chicks per group	150	150	150
Age of chicks, days	15	15	15
The average weight of 1 chicken at the beginning of the study, g	426	432	443
The number of oocysts in the tested samples (mean value), No. in a single FoV			
In cecum	40.3 ± 5.3	36.7 ± 1.9	32.8 ± 2.3
In 20 samples of droppings	33.7 ± 1.9	29.3 ± 2.1	24.6 ± 2.3
After treatment			
Chickens died within 48 days (%)	59 (39.3%)	3 (2.0%)	5 (3.3%)
The number of oocysts in the tested samples (mean value), No. in a single FoV			
In cecum	43.6 ± 4.4	–	5.5 ± 1.4
In 20 samples of droppings	40.3 ± 3.8	–	4.9 ± 1.1
Intensive efficacy, %	–	98.0	96.7
Survival rate of chicks within 48 days, %	60.7	98.0	96.7
Average daily gain within 48 days, g	44	53	48
Feed consumption per 1 kg of weight gain within 48 days, kg	2.24	1.95	2.10
Live weight at slaughter, g	2,536	2,978	2,729

No. in a single FoV – number of oocysts in a single field of view.

It was found that in the test groups, after the use of "Robenidine" and "Sarucoxum 12%" in therapeutic doses, 3 and 5 chicks respectively died during the entire observation period (48 days). Thus, the survival rate of poultry in test groups was 98.0 and 96.7%, respectively. In the control group, the survival rate of broiler chicks was significantly lower – 60.7%.

The post-mortem examination of dead chicks of groups 1 and 2 revealed no lesions characteristic of eimeriosis in the internal organs and intestines.

Microscopy of cecum smears from dead birds of group 1 demonstrated no *Eimeria* oocysts, whereas single oocysts were found in group 2 broiler chicks (4–5 oocysts in a single field of view).

The intensive efficacy of "Robenidine" treatment was 98.0%, of "Sarucoxum 12%" – 96.7%. Thus, the therapeutic efficacy of "Robenidine" turned out to be higher than "Sarucoxum 12%".

The average daily gain in broiler chicks during the rearing period (48 days) in test groups 1 and 2 was 53 and 48 g, respectively, and feed consumption per 1 kg of live weight gain was 1.95 and 2.1 kg.

CONCLUSION

Monitoring tests performed in the plain and piedmont zones of the Republic (Khasavyurtovsky and Karabudakhkentsy raions) revealed a high *Eimeria* infection rate in floor-housed poultry. The prevalence was 81.6 and 82.4%, respectively.

The prevalence of infection in cage-housed poultry in the mountain zone and intermontane valley zone (Khunzakhsky and Gergebilsky raions) was 61.2 and 58.0%, respectively.

Morphology study of *Eimeria* detected in pathological samples (droppings, ceca and duodena) allowed

to identify them as *Eimeria tenella*, *Eimeria maxima*, *Eimeria mitis*, *Eimeria acervulina*.

As a result of a comparative assessment of eimeriocidal drugs in the setting of spontaneous eimeriosis in broiler chicks in the commercial poultry farm AO "Poultry farm "Makhachkalinskaya", 98.0% "Robenidine" effectiveness was established.

Daily treatment of chicks with "Robenidine" from the first day of life during the entire rearing period at a dose of 33 g per 1 ton of feedstuffs and removing the drug the diet 5 days before slaughter keeps the poultry free from the parasites. The survival rate of the young poultry under study during the observation period was 98.0%.

Thus, the therapeutic efficacy of "Robenidine" turned out to be higher than "Sarucoxum 12%". The commercial drug "Robenidine" can be used in poultry farms of the Republic of Dagestan for treating eimeriosis in broilers.

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Use of "ARRIAH-AviFluVac" vaccine in turkeys, geese and ducks

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ABSTRACT

"ARRIAH-AviFluVac" vaccine against H5 avian influenza was demonstrated to be effective for ducks, geese and turkeys both in the laboratory and production environment. When administered to ducks at 0.5; 1.0 and 1.5 cm^3, the vaccine provided 100%-effective protection of birds against the disease and death after challenge with the relevant high pathogenicity avian influenza virus of subtype H5N1, clade 2.3.4.4b. Singular 0.5–1.5 cm^3 inoculation induced formation of antibodies, which were detected in the hemagglutination inhibition test at the titres that ranged from 4.3 to 6.1 log_2. The vaccine facilitated 9–26-fold decrease in the virulent virus shedding by the ducks. Protection of turkeys vaccinated at the dose of 1.0 cm^3 was maintained at the level of 87.5% after challenge with high pathogenicity avian influenza virus of subtype H5N1, clade 2.3.4.4b. The vaccine induced formation of antibodies at the titres of 4.9 and 5.5 log_2 in turkeys after singular and double vaccination at the dose of 1.0 cm^3, respectively. It was demonstrated, that after double administration of 1.0 cm^3 of "ARRIAH-AviFluVac" vaccine, the post-vaccinal avian influenza antibody level exceeded 5.0 log_2 in 75.9–90.0% of the geese population. The most appropriate way of the vaccine use in turkeys, ducks and geese involves at least its double administration at the double commercial dose. Higher species resistance of ducks to the challenge with avian influenza virus of subtype H5, clade 2.3.4.4b as compared to turkeys was also demonstrated.

Keywords: high pathogenicity avian influenza, H5 avian influenza virus, inactivated vaccine, immunogenicity, ducks, geese, turkeys

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Применение вакцины «ВНИИЗЖ-АвиФлуВак» для индеек, гусей и уток

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РЕЗЮМЕ

В лабораторных и производственных условиях показано, что вакцина против гриппа птиц H5 «ВНИИЗЖ-АвиФлуВак» является эффективным препаратом для профилактики болезни у гусей, уток и индеек. Вакцина при введении уткам в дозах 0,5; 1,0 и 1,5 $см^3$ со 100%-й эффективностью защищала птиц от заболевания и гибели при заражении актуальным вирусом высокопатогенного гриппа птиц подтипа H5N1 генетической клады 2.3.4.4b. Однократная прививка в дозе от 0,5 до 1,5 $см^3$ вызывала образование антител, выявляемых в реакции торможения гемагглютинации, в титрах от 4,3 до 6,1 log_2. Вакцина способствовала снижению выделения вирулентного вируса утками в 9–26 раз. Протективная защита индеек, привитых в дозе 1,0 $см^3$, обеспечивалась на уровне 87,5% при заражении вирусом высокопатогенного гриппа птиц подтипа H5N1 клады 2.3.4.4b. Вакцина вызывала образование антител в титрах 4,9 и 5,5 log_2 у индеек при однократном и двукратном введении в дозе 1,0 $см^3$ соответственно. Установлено, что при двукратном применении препарата «ВНИИЗЖ-АвиФлуВак» в дозе 1,0 $см^3$ уровень поствакцинальных антител к вирусу гриппа птиц был выше 5,0 log_2 у 75,9–90,0% популяции гусей. Рациональным решением использования вакцины для индеек, уток и гусей является ее применение в двойной коммерческой дозе и как минимум двукратное введение. Также была установлена более высокая видовая устойчивость уток к заражению вирусом гриппа птиц подтипа H5 клады 2.3.4.4b по сравнению с индейками.

Ключевые слова: высокопатогенный грипп птиц, вирус гриппа птиц H5, инактивированная вакцина, иммуногенность, утки, гуси, индейки

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INTRODUCTION

Avian influenza (AI) is a highly contagious viral disease that can affect several poultry species [1]. Over the past 10 years, high pathogenicity avian influenza (HPAI) H5Nx viruses of Eurasian genetic lineage Gs/Gd/96 clade 2.3.4.4 spread globally. In 2022–2023, an unprecedented HPAI panzootic occurred, which was caused by H5N1 virus of Eurasian genetic lineage 2.3.4.4b [2]. A huge number of the disease outbreaks in poultry and cases of the virus detection in wild and synanthropic birds of different species, as well as their mass mortality were reported in different countries. The virus was detected in terrestrial and marine mammals thus raising serious global concern due to pandemic threat (WOAH Situation Reports for Avian Influenza, 2021–2023; FAO Empres-i; WHO).

Starting from 2021, HPAI epizootics in the Russian Federation were also caused by H5N1 virus of Eurasian genetic lineage (clade) 2.3.4.4b [3].

Since the disease causes enormous damage to poultry farming and becomes enzootic in many countries, issues on the use of vaccination as an additional tool to contain the spread of infection and reduce unjustified losses were actively discussed at the international level in 2022–2023 [4, 5, 6]. The Terrestrial Animal Health Code (WOAH, Chapter 10.4) contains recommendations on poultry vaccination against HPAI and describes the conditions under which it can be used [7].

In most countries where AI vaccination is practiced, mainly whole-virion vaccines based on low-pathogenicity and genetically modified low-pathogenicity AI viruses obtained by reverse genetics and containing a hemagglutinin gene fragment of the relevant highly virulent H5 and H7 viruses are used.

Current inactivated AI vaccines are limited in effectiveness in *Anseriformes*, therefore, it is recommended to administer a double chicken dose, or to add a strong stimulant for effective immunity [8, 9, 10, 11]. It was also demonstrated that double dose of the bivalent vaccine protected ducks from the disease and death, but, however, the antibodies were formed at a low level (from 4 to 8 $\log_2$) and after challenge, the virus was isolated from 13% of vaccinated and 100% of unvaccinated birds. The inability of the challenge virus to induce the repeated production of antibodies in birds vaccinated with a closely related H5 strain is a convincing evidence of the lack of the virulent virus replication in the vaccinated ducks [12]. Most scientific articles show that the whole-virion vaccines are generally effective for ducks [9, 10, 13, 14, 15] and geese [10, 16], but these avian species react to vaccination in different ways [10, 17].

According to A. Kandeil et al., use of inactivated AI H5 vaccine induced development of an immune response in all avian species housed together on the backyards (ducks, geese and chickens), and after double vaccination the antibody titres reached 10 $\log_2$. The immune response was, however, different in different bird species. The vaccinated birds remained alive after challenge and shed less virus as compared to the unvaccinated ones. It should be noted that the unvaccinated ducks also did not get diseased and survived throughout the experiment. Moreover, the vaccinated ducks shed more virus than vaccinated birds of other species [10].

There are also many reports of positive results of genetically engineered vaccines used for HPAI prevention in poultry. Thus, according to E. Niqueux et al., simultaneous immunization with two recombinant Newcastle disease and fowlpox virus-vectored vaccines provided 12-week protection in Muscovy ducks [18]. Kim D. H. et al. also demonstrated that administration of genetically engineered Newcastle disease virus-vectored vaccine effectively protected Muscovy ducks from infection with the virulent HPAI H5 virus and decreased the virus shedding [19].

Most of the AI vaccine trials, both in the laboratory and in the field, are carried out in chickens and turkeys, because high mortality and excretion of a large amount of the virus into the environment are reported in them when infected. However, with the spread of AI in Asia, the disease epizootology changed, as indicated by the increased susceptibility of wild and exotic birds. Infection of domestic ducks and geese seriously affected the maintenance and spread of H5N1 AI [20].

In 2022, the Federal Centre for Animal Health (ARRIAH) registered "ARRIAH-AviFluVac" vaccine against AI (H5) based on the low-pathogenic H5 AI virus strain Yamal.

As part of the post registration process, studies were conducted to determine the vaccination dose and frequency of vaccination for various poultry species (turkeys, ducks and geese) in laboratory and field conditions, the results of which are demonstrated in this paper.

MATERIALS AND METHODS

Vaccine. "ARRIAH-AviFluVac" inactivated emulsion vaccine against avian influenza (H5) manufactured by the Federal Centre for Animal Health (batch 010122, release date 01.2022) was used in laboratory and field trails. The vaccine is prepared from the extraembryonic fluid of the chicken embryonated eggs infected with avian influenza virus (the source of H5N1 production strain Yamal is A/wildduck/YaNAO/956-21 isolate) inactivated with

aminoethylethanolamine supplemented with Montanide ISA 70 VG oil adjuvant (SEPPIC, France) at 30:70 w/w.

Poultry. Commercial poultry delivered from farms free from acute infectious avian diseases were used for laboratory tests: 20 day-old ducklings, 140 ducklings at the age of 21 days and 40 turkeys at the age of 10 days.

During the field trials, the vaccine was administered to commercial 1–28-day-old turkeys on one of the poultry farms in the Stavropol Krai and to the parent 30–60-day old goose flock in the Republic of Bashkortostan.

The experiment design at the animal keeping facilities. The day-old ducklings were divided into two groups of 10 birds in each. They were intramuscularly vaccinated at the doses of 0.25 and 0.5 cm^3, respectively; 28 days post immunization, the ducklings' blood sera were collected and tested for antibodies to AIV H5.

Twenty one-day-old ducklings were divided into 4 groups of 35 birds in each. The birds in group 1 were vaccinated with "ARRIAH-AviFluVac" vaccine at the dose of 0.5 cm^3; in group 2 – at the dose of 1.0 cm^3; in group 3 – at the dose of 1.5 cm^3. All ducklings were vaccinated intramuscularly into the thigh. The birds in group 4 were not vaccinated. After days 7, 14 and 21, blood samples were collected from the ducklings in each group to monitor seroconversion to AIV H5. Twenty eight days post vaccination, the birds in the experimental groups of 10 birds in each were challenged with the virulent influenza A H5 virus A/chicken/Stavropol/2077-6/21 H5N1 at the dose of 6.0 lg EID_{50} intramuscularly into the thigh at the volume of 0.5 cm^3. The challenged ducklings were subjected to 10-day clinical observation.

In 6 days post challenge, the oropharyngeal swabs were collected from the ducklings to identify the AIV genome by polymerase chain reaction (PCR).

Ten-day-old turkeys were divided into 4 groups of 10 birds in each. Poultry in the three experimental groups were intramuscularly vaccinated at the doses of 0.25; 0.5 and 1.0 cm^3, respectively. The turkeys in group 4 were not vaccinated. In 21 days post vaccination, blood was collected from the birds for testing for AIV H5 antibodies by hemagglutination inhibition test (HI). The turkeys were then challenged with A/chicken/Stavropol/2077/6/21 H5N1 virus at the dose of 6.0 lg EID_{50} intramuscularly into the thigh at the volume of 0.5 cm^3. The challenged birds were clinically observed for 10 days.

The experiment design in the field. An experimental group of 700 day-old turkeys was vaccinated at the dose of 0.2 cm^3. Then, at the age of 28 days the turkeys were divided into 2 groups of 350 birds in each and they were re-immunized at the doses of 0.5 and 1.0 cm^3, respectively.

Two groups of 30 geese were formed. The birds in group 1 were intramuscularly vaccinated once at the age of 30 days at the dose of 1.0 cm^3; the birds in group 2 were also intramuscularly vaccinated twice at the age of 30 and 60 days at the dose of 1.0 cm^3.

All experiments on birds were conducted in strict compliance with the interstate standards for animal handling and housing adopted by the Interstate Council for Standardization, Metrology and Certification, as well as in accordance with the requirements of Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes.

Serological tests for AIV H5 antibodies were carried out by HI test using the ARRIAH-manufactured HI test-kit for detection of antibodies against H5 avian influenza virus. HI results were expressed in log_2, and an antibody titre equal to or higher than 5 log_2 was considered to be the minimum protective titre according to the recommendations of the World Organization for Animal Health [21].

Challenge. Resistance of vaccinated poultry to the challenge with A/chicken/Stavropol/2077/6/21 H5N1 AIV at the dose of 6.0 lg EID_{50} was justified by the absence of mortality and disease clinical signs (depression, respiratory and nervous disorders) in the poultry.

Molecular genetic tests. Detection of AIV genome in biomaterial samples and determination of the amplification cycle threshold were carried out in accordance with the "Methodological recommendations for detection of Type A avian influenza virus RNA by real-time RT-PCR"[1].

Oropharengeal swabs were collected with sterile tupfers. The samples were collected from all ducklings in the relative groups in 6 days post challenge. Amplification cycle thresholds (*Ct*) were determined in the control group (Ct_0) and in the groups of the vaccinated birds (Ct_i). The reaction was considered positive (AIV H5 genome is present) if $0 < Ct < 37$ [21]. Next, a comparison with the control was performed and the difference of the compared values was calculated ($d_i = Ct_i - Ct_0$). In addition, the average estimates of the difference (*D*) and standard errors of measurement of the averages (± *m*) were calculated from the group samples (*d*).

The immunogenicity of "ARRIAH-AviFluVac" vaccine for ducks was determined in laboratory experiments by the results of the resistance to challenge, by the titres of serum antibodies and by PCR-indicated virus shedding.

The immunogenicity of "ARRIAH-AviFluVac" vaccine for turkeys was determined in laboratory and field experiments based on the results of the resistance to challenge and serum antibody titres.

The immunogenicity of "ARRIAH-AviFluVac" vaccine for geese was determined in the field experiments by serum antibody titres.

Statistical processing of the resulted data included determination of mean values, error of mean, statistical significance of the differences between the experimental groups of animals along with the indication of the statistical criterion values, number of degrees of freedom and prediction error probability (*p*).

RESULTS AND DISCUSSION

Table 1 demonstrates the HI results of the sera of ducklings of different ages tested for the antibodies to AIV H5 after vaccination.

It was found that day-old ducklings vaccinated at the dose of 0.25 cm^3 demonstrated AIV H5 antibodies in low nonuniform titres ($p > 0.05$). At the same time, the AIV H5 antibody titres in the group of ducklings vaccinated at the dose of 0.5 cm^3 reached 4.4 log_2 with good uniformity in 28 days ($p \leq 0.001$).

The vaccinated 21-day-old ducklings demonstrated AIV H5 antibodies in comparable titres regardless of the vaccine dose (0.5; 1.0 or 1.5 cm^3). Seroconversion

[1] Andriyasov A. V., Andreychuk D. B., Chvala I. A. Methodological recommendations for detection of Type A avian influenza virus RNA by real-time RT-PCR: No. 45-16. Vladimir: ARRIAH; 2016. 13 p. (in Russ.)

Table 1
Results of duckling serum tests for AIV H5 antibodies using HI test

Age of birds at the time of vaccination, day	Vaccination dose, cm^3	Antibody titres (log_2) at different time points post vaccination			
		Day 7	Day 14	Day 21	Day 28
1	0.25	n/t	n/t	n/t	1.3 ± 0.6
	0.5	n/t	n/t	n/t	4.4 ± 0.3
21	0.5	0.9 ± 0.6*	0.9 ± 0.6 (0)	4.3 ± 0.5 (4,5)	4.4 ± 0.6
	1.0	3.1 ± 0.6	3.1 ± 0.6 (4.0)	5.0 ± 0.4 (5.0)	5.1 ± 0.4
	1.5	2.1 ± 0.8 (1.5)	2.1 ± 0.8 (1.5)	6.1 ± 0.5 (6.0)	6.2 ± 0.6

* mean HI antibody titre and error, median (Me) is in brackets; n/t – not tested.

was observed in groups of immunized ducklings 7 days later with antibody titres reaching the maximum levels in 28 days post vaccination.

Statistical processing of the primary antibody titres obtained 28 days post vaccination in the experimental groups was carried out by one-way analysis of variance in Excel. It was found that the F-test statistics was below F critical value ($F_{test}3.0 < F_{critical}3.4$), that is, the mean antibody titres for the three groups did not differ.

To determine the protective properties of the vaccine against the H5 HPAI pathogen, the vaccinated ducks were challenged with A/chicken/Stavropol/2077-6/21 H5N1 virus. Table 2 demonstrates the results of the experiment.

It was found that the vaccinated ducklings in all experimental groups did not get diseased within 10 days after challenge with the high pathogenicity AI H5N1 virus. In the group of the unvaccinated birds, six birds demonstrated the disease signs and one bird died.

In order to establish the immunity level in the vaccinated ducks, the studies were performed to detect the virulent virus shedding. This stage was targeted at the detection of AIV H5 genome in the oropharyngeal secretions of the birds in 6 days after the challenge.

It was revealed that AIV H5 genome was present in all tested samples. However, the initial concentration of the viral material in the oropharyngeal excretions of the vaccinated birds was significantly lower as compared to the controls. Thus, amplification cycle thresholds (*Ct*) in the control group ranged from 20.93 to 25.47 (average – 23.83), in the group of ducklings vaccinated at the dose of 0.5 cm^3 – from 24.49 to 29.46 (average – 26.84), in the group of ducklings vaccinated at the dose of 1.0 cm^3 – from 23.08 to 30.29 (average – 27.5), in the group of ducklings vaccinated at the dose of 1.5 cm^3 – from 26.12 to 31.41 (average – 28.32). Considering that one amplification cycle approximately involves doubling of the amount of the target product, the initial concentration of the viral material in the test sample (%) as compared to the control can be described as $J = (1/2^D) \times 100$. Hence, by the groups of the vaccinated birds, the target estimates were (%): $J_I = 10.8$; $J_{II} = 6.9$ and $J_{III} = 3.9$, i.e. the ducks vaccinated at the dose of 0.5 cm^3 shed the virus in 9-fold lesser amount, at the dose of 1.0 cm^3 – in 14-fold lesser amount, and at the dose of 1.5 cm^3 – in 26-fold lesser amount as compared to the unvaccinated birds.

The vaccination of ducklings, therefore, contributed to the decrease of the virus shedding by the vaccinated birds compared with the unvaccinated ones, and the higher the vaccine dose, the more significant the decrease was.

To determine the protective properties of the vaccine against the AIV H5 pathogen, the vaccinated turkeys were infected with A/chicken/Stavropol/2077-6/21 H5N1 virus in the laboratory.

The data presented in Table 3 demonstrate that the resistance of the vaccinated turkeys to infection varied in different groups. Thus, turkeys vaccinated at the dose of 1.0 cm^3 (87.5%) had the highest resistance, and the turkeys vaccinated at the dose of 0.25 cm^3 (28.6%) had the lowest resistance. The unvaccinated turkeys died in 3 days post infection.

Postvaccinal humoral immune response was determined in turkeys before challenge. It was found that in the group of birds vaccinated at the dose of 0.25 cm^3, the average antibody titre was 3.3 ± 0.6 log_2, in the group vaccinated at the dose of 0.5 cm^3 – 4.0 ± 0.6 log_2, and in the group vaccinated at the dose of 1.0 cm^3 – 4.9 ± 0.4 log_2.

Thus, single vaccination of turkeys at the dose of 1.0 cm^3 induced the most intense immune response to AIV H5, which was expressed by high protection level (87.5% of the birds did not get diseased) and high antibody titres.

In addition to the laboratory tests, field trials of the vaccine were carried out in commercial turkeys in the Stavropol Krai.

The turkey immunity level to AIV H5 was assessed by HI antibody titres in 35 days after the second vaccination.

Table 2
Resistance of vaccinated ducklings to challenge with subtype H5N1 influenza A virus

Observation period, days	Groups by the inoculation dose, cm^3			
	0.5	1.0	1.5	Control
1	0/10*	0/10	0/10	0/10
2	0/10	0/10	0/10	0/10
3	0/10	0/10	0/10	0/10
4	0/10	0/10	0/10	0/10
5	0/10	0/10	0/10	2/10
6	0/10	0/10	0/10	5/9 (1 dead)
7	0/10	0/10	0/10	5/9
8	0/10	0/10	0/10	5/9
9	0/10	0/10	0/10	5/9
10	0/10	0/10	0/10	5/9

* ratio between the diseased ducklings to the total number of duckling in the group.

Table 3
Resistance of vaccinated turkeys to challenge with subtype H5N1 influenza A virus

Observation period, days	Groups according to the vaccination dose, cm^3			
	0.25	0.5	1.0	Control
1	0/7*	0/9	0/8	0/8
2	0/7	0/9	0/8	2/8
3	0/7	0/9	0/8	6/6
4	1/7	0/9	0/8	–
5	2/6	1/9	0/8	–
6	1/4	2/8	0/8	–
7	1/3	1/6	1/8	-
8	0/2	0/5	0/7	–
9	0/2	1/4	0/7	–
10	0/2	0/4	0/7	–
Protection level, %	28.6 (2/7)**	44.4 (4/9)	87.5 (7/8)	0 (0/8)

* ratio between the dead birds to the total number of birds in the group;
** ratio between the survived birds to the total number of birds in the group.

It was found that the average antibody titres in the group of turkeys vaccinated twice at the dose of 1.0 cm^3 were 5.5 ± 0.2 log_2, and in the group vaccinated at the dose of 0.5 cm^3 – 3.5 ± 0.3 log_2. Statistical results differed with high degree of confidence (99.9%).

Based on the results of the laboratory tests and field trials of "ARRIAH-AviFluVac" vaccine in turkeys, the optimal parameters of its administration were established, i.e.: dose – 1.0 cm^3, frequency – at least double vaccination.

Studies were also conducted to examine the vaccine immunogenicity in commercial geese in the Republic of Bashkortostan.

The data in Table 4 demonstrate that after single immunization, the vaccine induced formation of AIV H5 antibodies in 48.5% of the goose population at the titre of 3.7 log_2 one month after vaccination; and in two months after immunization, the number of birds with protective antibody titres was only 23.3% and the average group titre was 1.9 log_2.

As the study results demonstrated, the minimum protective AIV H5 antibody titre (≥ 5 log_2) after double vaccination was reported in 22–27 out of 30 birds for 10 months, i.e. the protection coverage was at the level of 75.9–90.0% of the population. During this period, the average antibody titres were also at a high level and ranged from 6.3 to 6.8 log_2.

CONCLUSION

Single vaccination of ducklings with "ARRIAH-AviFluVac" vaccine at the dose of 0.5–1.5 cm^3 induced HI-detected formation of antibodies in titres from 4.3 to 6.1 log_2, which is consistent with the data reported by D. Middleton [12]. It was demonstrated that the ducklings were resistant to infection with the AIV H5N1 28 days after single administration of "ARRIAH-AviFluVac" vaccine at doses of 0.5, 1.0 and 1.5 cm^3. It is also worth mentioning that the initial concentration of the viral material in the oropharyngeal excreta of the vaccinated birds was 9–26 times lower as compared to the unvaccinated birds.

Thus, the vaccine has a high antigenicity sufficient for the formation of immunity in ducklings when administered at the doses from 0.5 to 1.5 cm^3. It was also demonstrated that inoculation of day-old ducklings at the dose of 0.25 cm^3 was insufficient to protect the birds.

The unvaccinated ducklings were less susceptible to the infection with the virulent H5 virus of clade 2.3.4.4b as compared to turkeys, and 60% of the birds were diseased, which is consistent with the data on low sensitivity of unvaccinated ducks when infected with the H5 virus of clade 2.2.1.2 H5N1 reported by A. Kandeil et al. [10]. This is indicative of the capacity of the virus carrier ducks to maintain the pathogen reservoir.

The laboratory study results demonstrated that the optimal inoculation dose of "ARRIAH-AviFluVac" vaccine for turkeys was 1.0 cm^3, when the vaccinated birds were protected from the infection by 87.5% and antibodies were formed at the highest titres (4.9 log_2).

In the field trials, the vaccine effectiveness was also demonstrated when used twice at the dose of 1.0 cm^3, when it was possible to gain high antibody titres (5.5 log_2) in commercial turkeys. Based on the data resulted from the laboratory and field trials, it was found that the dose of 1.0 cm^3 is optimal for use in turkeys, and the frequency of vaccination should be at least double vaccination.

In the field trials, two schemes of "ARRIAH-AviFluVac" administration were tested in geese, and it was found that when administered twice at the dose of 1.0 cm^3 the vaccine provided 10-month immunity in 75.9–90.0% of the poultry population.

The data obtained are consistent with the conclusions of a number of scientists [8, 9, 10, 11] and indicate that double administration of "ARRIAH-AviFluVac" vaccine against AI H5 at a double commercial dose and at least twice is reasonable for large and domestic waterfowl species with subsequent control of immunity level and revaccination by indications.

Table 4
Results of goose serum tests for post-vaccinal AIV H5 antibodies

Vaccination frequency	Antibody titres (log_2) at different time points after vaccination				
	day of vaccination	1 month	2 months	7 months	10 months
Single	n/d	3.7 ± 0.6 (16/33*; 48.5%)	1.9 ± 0.4 (7/30; 23.3%)	n/d	n/d
Double	0.8 ± 0.3	6.3 ± 0.5 (25/30; 83.3%)	n/d	6.5 ± 0.5 (22/29; 75.9%)	6.8 ± 0.3 (27/30; 90.0%)

* seroconversion is expressed as the ratio between the number of birds demonstrating HI antibody titre above 5 log_2 and total number of tested birds (%);
n/d – no data.

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Overview of animal infectious disease situation in the Republic of Dagestan in 2023

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ABSTRACT
An epizootiological survey of livestock farms of Dagestan was conducted, the main infectious diseases common in the region were considered, and measures taken to protect against them were described. At present, the Veterinary Service is undertaking systematic efforts to prevent the occurrence and spread of infectious diseases such as brucellosis, leukosis, rabies, pasteurellosis, blackleg, bradsot and enterotoxemia in the Republic. Among the above-mentioned diseases reported in 2023, brucellosis and leukosis are responsible for the vast majority of outbreaks and diseased animals detected in them. In particular, the following diseases have the largest share in the nosological profile of quarantinable infectious diseases based on the number of detected infected localities during the period under study: brucellosis (52.63%), bovine leukosis (30.70%), rabies (8.77%), enterotoxemia (3.51%), pasteurellosis (1.75%), bradsot (1.75%) and blackleg (0.88%). In total, 1,812 animals were affected with quarantinable infections and 35 animals died in 114 infected localities. Most often over the past year, quarantinable infections were reported in cattle (69.59% of cases) and small ruminants (29.36%); in 1.05% of cases, the diseases affected horses, cats, wild animals and birds. In order to maintain animal disease freedom and sustainable growth of livestock production, the Veterinary Committee of the Republic of Dagestan annually implements measures to prevent the occurrence and spread of 75 diseases of animals and birds, including 10 highly dangerous ones. Anti-epizootic measures taken in the past year included a total of 93.8 million vaccinations and 6.2 million tests performed in the diagnostic institutions. Plans for the prevention of highly dangerous and other contagious diseases of animals and birds were fully implemented.

Keywords: monitoring, animal disease situation, Republic of Dagestan, infectious diseases, quarantinable diseases, infected locality, disease outbreak

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Обзор эпизоотической ситуации по инфекционным болезням животных в Республике Дагестан в 2023 году

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РЕЗЮМЕ
Проведено эпизоотологическое обследование животноводческих хозяйств Дагестана, рассмотрены основные инфекционные заболевания, характерные для региона, описаны мероприятия, проводимые для защиты от них. На данный момент ветеринарной службой ведется систематическая работа по предотвращению возникновения и распространения в республике таких инфекционных заболеваний, как бруцеллез, лейкоз, бешенство, пастереллез, эмкар, брадзот и энтеротоксемия. Среди вышеперечисленных заболеваний, зарегистрированных в 2023 г., подавляющее большинство эпизоотических очагов и выявленных в них заболевших животных приходится на бруцеллез и лейкоз. Так, в нозологическом профиле карантинных инфекционных заболеваний наибольший удельный вес по числу выявленных неблагополучных пунктов за исследуемый период занимают: бруцеллез (52,63%), лейкоз крупного рогатого скота (30,70%), бешенство (8,77%), энтеротоксемия (3,51%), пастереллез (1,75%), брадзот овец (1,75%) и эмфизематозный карбункул (0,88%). Всего в 114 неблагополучных пунктах карантинными инфекциями заболело 1812 и пало 35 животных. Чаще всего за истекший год карантинные инфекции регистрировали среди крупного (в 69,59% случаев) и мелкого (29,36%) рогатого скота, в 1,05% случаев болезни поражали лошадей, кошек, диких животных и птиц. Для сохранения эпизоотического благополучия и устойчивого роста производства животноводческой продукции Комитетом по ветеринарии Республики Дагестан ежегодно проводятся профилактические мероприятия по предупреждению возникновения

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и распространения 75 заболеваний животных и птиц, из которых 10 являются особо опасными. Общий объем противоэпизоотических мероприятий в истекшем году составил 93,8 млн головообработок и 6,2 млн исследований в диагностических учреждениях. Планы по профилактике особо опасных и других заразных заболеваний животных и птиц выполнены в полном объеме.

Ключевые слова: мониторинг, эпизоотическое состояние, Республика Дагестан, инфекционные заболевания, карантинные заболевания, неблагополучный пункт, очаг заболевания

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INTRODUCTION

Studying an epizootic process plays a major role in the development of effective anti-epizootic measures for infectious disease control. A set of measures aimed at the eradication of infection outbreaks and the prevention of pathogen introduction into disease free farms should take into account the specificities of krais and regions [1, 2].

Maintaining sustainable freedom of the country's livestock from quarantinable infectious diseases is the most important task of veterinary science and practice, it is crucial in protecting the health and life of humans and animals, in providing the population with ecologically safe food products and industry with high-quality raw materials [3, 4, 5].

Therefore, anti-epizootic measures should be developed taking into account the knowledge of the animal disease situation based on the analysis of long-term data on epizootic process development for each infectious disease in a particular area. An effective solution to this problem requires appropriate information support, the establishment and implementation of an epizootological monitoring system [6, 7, 8, 9, 10, 11].

Disease freedom of a particular animal population is achieved through the implementation of a set of special, general and organizational measures aimed at the effective control of the epizootic process, the main condition of which is the commitment to achieve biological equilibrium in any parasitic systems in all cases at the territorial and population levels [12, 13].

Dagestan Republic is one of the largest regions in the North Caucasus. Its total area is 50.3 thousand square kilometres. It occupies the northeastern slopes of the Main Caucasian Ridge and the southwestern part of the Caspian Depression. The territory of the Republic is divided into four main physico-geographical zones: flatland, foothill, mountain and highland, based on which the zonal classification of municipal areas and urban districts was approved by a Decree of the Government of Dagestan Republic[1].

Animal husbandry occupies a leading place in the agriculture of Dagestan Republic and is represented by the following sectors of great importance: cattle, sheep, pig, horse, poultry farming, beekeeping, pond fish farming and others. The most essential conditions for their successful development are the prevention and eradication of quarantinable infectious diseases, which cause significant economic damage resulting from animal deaths, reduced performance and product quality, as well as the costs of basic organizational-economic, veterinary-sanitary and anti-epizootic measures. In the light of the above, studying the animal and bird infectious disease situation in Dagestan Republic is currently important and serves as the basis for the improvement of regional programmes aimed at animal contagious disease eradication.

The aim of the study is to monitor and assess the quarantinable animal and bird infectious disease situation in Dagestan Republic in 2023.

MATERIALS AND METHODS

The examination and analysis of statistical reports were carried out at the Laboratory of Infectious Pathology of Caspian Zonal Research Veterinary Institute – Branch of the Federal Agrarian Scientific Center of Dagestan Republic and in the administrative districts.

The quarantinable animal infectious disease situation was examined by analyzing data from the veterinary reports of the Veterinary Committee of Dagestan Republic, the republican, inter-raion and raion veterinary laboratories, the municipal and raion veterinary departments.

The epizootological survey of livestock establishments located in different physical and climatic zones and of individual settlements and farms was carried out in accordance with "Methodical guidelines for epizootological study"[2] and "Recommendations on epizootological study methods"[3].

The data were statistically processed and analysed using generally accepted methods [14].

[1] On approval of the zonal classification of municipal areas and urban districts of Dagestan Republic: Decree of the Government of Dagestan Republic No. 48 of 11 March 2019. https://docs.cntd.ru/document/553164815 (in Russ.)

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Table 1
Data on animal and bird contagious diseases reported in Dagestan Republic in 2023

Disease	Species	During the reporting period				Ongoing as of the end of the reporting period	
		number of detected infected localities	outbreaks			total number of infected localities	total number of outbreaks
			number of detected outbreaks	number of diseased animals/birds	number of dead animals/birds		
Brucellosis	cattle	48	117	1,236	–	71	252
	small ruminants	12	13	500	–	9	10
	horses	–	–	1	–	–	–
Leukosis	cattle	35	110	18	–	96	214
Rabies	cattle	4	4	4	4	–	–
	cats	3	3	3	3	–	–
	wild animals	3	3	3	3	–	–
Pasteurellosis	cattle	–	2	2	2	–	1
	small ruminants	2	4	5	5	–	–
Blackleg	cattle	1	1	1	1	1	1
Bradsot	small ruminants	2	2	4	4	–	–
Enterotoxemia	small ruminants	4	4	7	7	–	–
Dysentery	small ruminants	–	–	1	1	–	–
Epididymitis	small ruminants	–	–	15	–	6	6
Dourine	horses	–	5	7	–	–	5
Avian influenza	birds	–	1	5	5	–	–
Total	–	114	269	1,812	35	183	489

RESULTS AND DISCUSSION

The priority areas of the activities of the State Veterinary Service are maintaining the animal disease freedom of the region, ensuring the production of veterinary-safe animal products and protecting people from diseases shared between humans and animals.

The data on the animal and bird infectious disease situation in Dagestan Republic for the period under study are presented in Table 1.

It was found that a total of 114 quarantinable animal infectious disease infected localities were reported in the region in 2023, of which 60 were for brucellosis in cattle and small ruminants, 35 for bovine leukosis, 10 for rabies in domestic and wild animals, 4 for enterotoxemia in sheep, 2 for pasteurellosis, 2 for bradsot and 1 for blackleg.

The following diseases have the largest share in the nosological profile of quarantinable infectious diseases (Fig.) based on the number of detected infected localities during the period under study: brucellosis (52.63%), bovine leukosis (30.70%), rabies (8.77%), enterotoxemia (3.51%), pasteurellosis (1.75%), bradsot (1.75%) and blackleg (0.88%). In total, 1,812 animals became diseased in 114 localities infected with quarantinable infections.

Over the past year, quarantinable infections were most often reported in cattle (in 69.59% of cases) and small ruminants (29.36%); in 1.05% of cases, the diseases affected horses, cats, wild animals and birds (Table 2).

During the monitoring period, 35 animals died of infectious diseases in Dagestan Republic; in particular, 28.57% of domestic and wild animals died of rabies, 20.0% of cattle and small ruminants died of pasteurellosis, 20.0% of sheep died of enterotoxemia, 11.43% of sheep died of bradsot; 14.29% of birds died of influenza, 2.86% of cattle died of blackleg, and 2.86% of sheep died of dysentery.

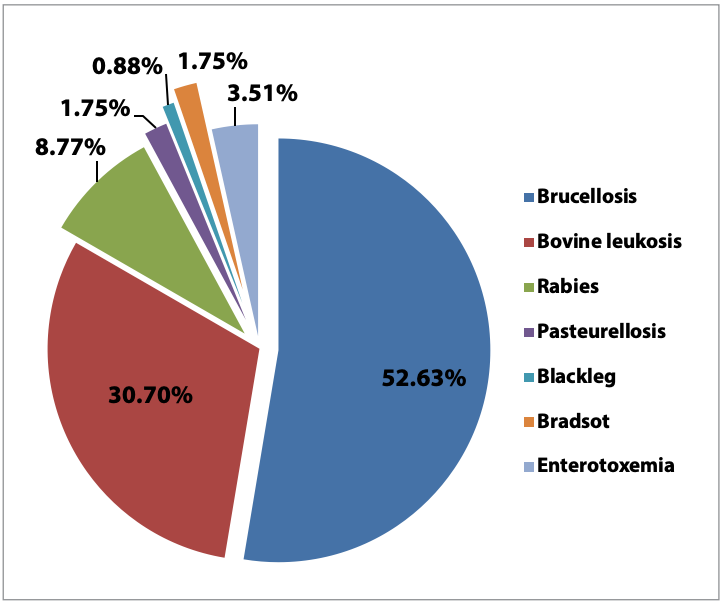

Fig. Nosological profile of quarantinable infectious diseases in Dagestan Republic in 2023

Table 2
Quarantinable infection occurrence in animals and number of infected localities in Dagestan Republic in 2023

Species	Infected localities		Diseased animals/birds	
	abs. number	%	abs. number	%
Cattle	88	77.19	1,261	69.59
Small ruminants	20	17.54	532	29.36
Horses	–	–	8	0.44
Cats	3	2.63	3	0.17
Wild animals	3	2.63	3	0.17
Birds	–	–	5	0.28
Total	114		1,812	

For many years, Dagestan has been a region where brucellosis persists in cattle and small ruminants [15]. In the past year, 48 bovine brucellosis infected localities and 12 small ruminant brucellosis infected localities were reported, 117 and 13 outbreaks were detected, with the diseased animals in them representing, respectively, 68.21 and 27.59% of the total number of animals affected with quarantinable diseases. In order to improve the situation, the Veterinary Service of the Republic implements a set of veterinary-sanitary and organizational-economic anti-brucellosis measures. In 2023, 1,210.492 thousand cattle, 494.238 thousand small ruminants and 22.091 thousand horses were examined, of which 1,236; 500 and 1 animals, respectively, were found to be brucellosis positive. Vaccination against brucellosis covered 830.735 thousand cattle and 3,284.252 thousand small ruminants. The disease eradication measures for infected holdings, establishments and farms are implemented within the framework of the overall set of the disease control activities, including the culling of reactor animals based on the diagnostic test results and the simultaneous use of anti-brucellosis vaccines to ensure immune protection.

One of the most common infectious diseases causing significant economic damage is viral leukosis, which has been reported in dairy cows and young animals in the majority of municipal entities of Dagestan Republic for many years [16, 17, 18]. To detect the disease, a serological method such as agar gel immunodiffusion (AGID) test is used. In 2023, the Veterinary Service of the region conducted AGID tests of 1,104.244 thousand cattle; 2,778 animals (0.25% of the tested cattle) were found to be the virus carriers. It was established that in 25 rural areas, municipalities and transhumance zones, leukemia virus occurrence in animals was moderate (from 0.01 to 9.2%). Hematological tests detected 18 leukosis affected animals. During the period under study, 35 new leukosis infected localities were identified; the association of bovine leukosis with specific territories, namely the farms of the Babayurtovsky, Kizlyarsky, Kizilyurtovsky, Kumtorkalinsky, Tarumovsky raions and the city of Makhachkala, was noted. The implementation of measures provided for by the subprogramme "Prevention and eradication of bovine leukosis in the holdings of Dagestan Republic" of the governmental programme of Dagestan Republic "Development of agriculture and regulation of agricultural product, raw material and food markets"[4] made it possible to eradicate the infection on many farms.

Dagestan remains a rabies infected region [19, 20]. The rabies situation is challenging to a certain extent; during the period under study, the disease was reported in 10 infected localities: in cattle in 4 localities (Gunibsky, Tarumovsky, Khasavyurtovsky raions and the city of Makhachkala), in cats in 3 localities and in wild animals in 3 localities. In order to control the disease, monitoring tests and vaccination of susceptible animals are carried out in the region. In 2023, 4 wild animals, 5 cattle, 9 cats, 3 dogs were tested for rabies; 7,080 cats, 38,570 dogs, 118.311 thousand cattle, 725 horses and 21.210 thousand small ruminants were vaccinated.

Over the past year, 2 bradsot outbreaks were detected in the Republic, in which 4 sheep became diseased and died. Bradsot is an acute, often fulminant toxicoinfection of sheep and goats, which is characterized by sudden death of animals. In some cases, severe seizures, nervous manifestations are observed, and animals die within a few hours. A characteristic sign that provides grounds for bradsot suspicion is the hemorrhagic inflammation of the abomasum, which is detected by necropsy. In order to prevent bradsot, 872.144 thousand sheep were immunized on the farms of Dagestan.

In February 2023, high pathogenicity avian influenza (HPAI) outbreak was detected in the area of a natural body of water located 1 km northeast of the settlement of Solnechnoye (Khasavyurtovsky raion), where 5 swans became diseased and died. The diagnosis was confirmed by laboratory tests, and restrictive measures (quarantine) were imposed. Subsequently, no new death cases were reported in synanthropic, wild birds and poultry. In order to control the HPAI situation, 5 wild birds and 8,410 poultry were examined, 36,608 poultry were vaccinated against high pathogenicity avian influenza.

As for other quarantinable infectious diseases such as pasteurellosis, blackleg, enterotoxemia, dysentery, ovine epididymitis and dourine, single cases were reported, and the measures taken made it possible to contain the diseases and prevent economic damage.

In order to maintain animal disease freedom and sustainable growth of livestock production, the Veterinary Committee of Dagestan Republic annually implements measures to prevent the occurrence and spread of 75 diseases of animals and birds, including 10 highly dangerous ones.

Anti-epizootic measures taken in the past year included a total of 93.8 million vaccinations and 6.2 million tests performed in the diagnostic institutions. Plans for the prevention of highly dangerous and other contagious diseases of animals and birds were fully implemented.

CONCLUSION

The analysis of the study findings shows that animal infectious diseases mentioned in the paper are the main factors hindering the development of livestock farming

[4] On approval of the governmental programme of Dagestan Republic "Development of agriculture and regulation of agricultural product, raw material and food markets": Decree of the Government of Dagestan Republic No. 673 of 13 December 2013. https://docs.cntd.ru/document/422452925 (in Russ.)

in Dagestan Republic. The complete eradication of these infections is not yet possible. However, it is possible to monitor changes in the animal disease situation and reduce its tension, using real epizootological monitoring data for this purpose. The risk of animal infectious disease occurrence dictates the need for the implementation of the systematic monitoring and careful analysis of the animal disease situation. In 2023, 114 quarantinable animal disease infected localities were reported in Dagestan Republic, including 60 for brucellosis in cattle and small ruminants, 35 for bovine leukosis, 10 for rabies in domestic and wild animals, 4 for enterotoxemia, 2 for pasteurellosis, 2 for bradsot and 1 for blackleg. As of the end of the period under study, there were 183 infected localities, including 96 for bovine leukosis, 80 for bovine and small ruminant brucellosis, 6 for ovine epididymitis and 1 for blackleg. The presented data on the occurrence of quarantinable animal diseases contribute to the scientific and practical studies of the epizootiology of the main infectious diseases reported in Dagestan Republic.

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The effect of the nisin-based pharmaceutical formulation used in the treatment plan for cows with subclinical mastitis on the milk microbiota

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ABSTRACT
Due to the growing threat of antimicrobial resistance, the search and development of new drugs to treat infectious mammary gland diseases of high yielding cows is an urgent task. The paper presents data on the microbiota composition of milk from high yielding cows suffering from subclinical mastitis; 144 microbial isolates were recovered from 70 milk samples; with the highest number of *Staphylococcus aureus* and *Streptococcus dysgalactiae* detected (22.2 and 16.0%, respectively). The study showed that a significant number of *Staphylococcus aureus* isolates (53.1%) were resistant to I generation cephalosporins; 52.6% of the isolated *Streptococcus dysgalactiae* strains showed resistance to tetracyclines; 33.3% of *Staphylococcus haemolyticus* isolates were resistant to macrolides. 42.1; 35.3 and 62.5% of *Enterococcus faecium*, *Aerococcus viridans* and coliform bacteria isolates, respectively, were resistant to penicillins. 38.5% of *Staphylococcus epidermidis* isolates were found to be resistant to tetracyclines. *Corynebacterium pseudotuberculosis* isolates showed equal resistance to penicillin and tetracycline antimicrobials (20.0%). The research revealed presence of multi-drug resistant coliform bacteria, *Streptococcus dysgalactiae*, *Aerococcus viridans*, *Staphylococcus aureus* strains. Experiments to study the effect of the new nisin-based pharmaceutical formulation on microbiota of milk from cows with subclinical mastitis were carried out using 35 high yielding cows. A microbiological testing of cow milk on day 14 from the beginning of the treatment showed that the number of microbiota-free samples increased to 88.6%, while in 1.4% of cases *Staphylococcus aureus* isolates were recovered (10^3 CFU/mL). The titers of coliform and *Staphylococcus aureus* bacteria isolated in 1.4% (10^1 CFU/mL) and 2.7% (10^2 CFU/mL) of cases, respectively, were not etiologically significant.

Keywords: cows, subclinical mastitis, antimicrobial resistance, antimicrobials, treatment regimen, bacteriocin nisin, milk microbiota, colony-forming units

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Влияние композиции на основе бактериоцина низина в схеме лечения коров с субклиническим маститом на микробиоту молока

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РЕЗЮМЕ
В связи с растущей угрозой развития антибиотикорезистентности поиск и разработка новых средств для лечения инфекционных заболеваний молочной железы высокопродуктивных коров является актуальной задачей. В статье представлены данные по изучению состава микробиоты секрета молочной железы высокопродуктивных коров при скрытой форме мастита. Из 70 проб секрета молочной железы было выделено 144 изолята микроорганизмов, наибольшее количество приходилось на *Staphylococcus aureus* и *Streptococcus dysgalactiae* (22,2 и 16,0% соответственно). Исследованиями установлено, что у максимального количества изолятов *Staphylococcus aureus* (53,1%) наблюдали устойчивость к цефалоспоринам I поколения. Выделенные штаммы *Streptococcus dysgalactiae* в 52,6% случаев проявили устойчивость к препаратам группы тетрациклинов; 33,3% изолятов *Staphylococcus haemolyticus* были резистентны к препаратам группы макролидов. Устойчивостью к препаратам групп пенициллинов обладали 42,1; 35,3 и 62,5% изолятов *Enterococcus faecium*, *Aerococcus viridans* и бактерий группы кишечной палочки соответственно. В 38,5% случаев установлена резистентность к препаратам группы тетрациклинов у изолятов *Staphylococcus epidermidis*. Изоляты *Corynebacterium pseudotuberculosis* проявили устойчивость к антимикробным

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препаратам групп пенициллинов и тетрациклинов в равной степени (20,0%). Полученные данные показали наличие полирезистентных штаммов бактерий группы кишечной палочки, *Streptococcus dysgalactiae*, *Aerococcus viridans*, *Staphylococcus aureus.* Экспериментальные исследования по изучению влияния разработанной фармацевтической композиции, содержащей бактериоцин низин, на состав микробиоты молока при лечении коров с субклиническим маститом выполнены на 35 высокопродуктивных коровах. Проведенное на 14-й день с начала курса лечения микробиологическое исследование секрета молочной железы коров показало, что число проб с отсутствием микрофлоры увеличилось до 88,6%, при этом количество колониеобразующих единиц, равное 10^3 КОЕ/мл, установлено у 1,4% изолятов *Staphylococcus aureus.* Выделенные в 1,4 (10^1 КОЕ/мл) и 2,7% (10^2 КОЕ/мл) случаев бактерии группы кишечной палочки и *Staphylococcus aureus* соответственно не являлись этиологически значимыми в диагностическом титре.

Ключевые слова: коровы, субклинический мастит, антибиотикорезистентность, антимикробные препараты, схема лечения, бактериоцин низин, микробиота молока, колониеобразующие единицы

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INTRODUCTION

Mammary gland inflammation in cows is most often caused by a bacterial infection [1, 2, 3]. The quantity of bacteria detected depends on the form of mastitis and its severity, as well as the pathogen type [4, 5, 6, 7]. The most often detected microorganisms in milk of cows suffering from mastitis are *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae*, *Escherichia coli*, *Enterococcus faecium*, *Enterococcus faecalis* [1, 2, 3, 4]. The subclinical form of mastitis, which is more difficult to diagnose due to the lack of visible changes in the mammary gland and milk is the major danger for dairy farming, though it gives higher somatic cell counts and total bacterial counts in milk. Microbial metabolites and toxins accumulate in the milk from subclinically mastitic cows affecting milk taste, nutritional value of raw milk and dairy products and decreasing their shelf life [8, 9, 10, 11]. The prevalence of subclinical mastitis in high yielding cows in developing countries is high [12, 13, 14, 15, 16]. That is why to reduce milk rejection and prevent antimicrobial resistance (AMR), the use of antimicrobials is minimized in treatment plans, and vaccines, bacteriophages, phage lysines, bacteriocins are used as an alternative [17, 18, 19, 20, 21, 22, 23, 24]. Pursuant to the "Strategy to Prevent the Spread of Antimicrobal Resistance in the Russian Federation to 2030", approved by the Russian Federation Government Decree on 25 September 2017 No. 2045-r, we tested the drugs based on antimicrobial peptides of microbial origin used to treat infectious mammary gland diseases of high yielding cows.

The work is relevant because the formulation is based on bacteriocin nisin to be used in the treatment regimen for cows with subclinical mastitis as an alternative to known antimicrobial drugs.

The novelty of the work is that for the first time, data on the effect of a new nisin-based formulation used in the treatment regimen of cows with subclinical mastitis on the milk microbiota were obtained.

Practical significance: in order to prevent AMR, the use of the nisin-based formulation makes it possible to reduce the use of antimicrobial drugs for mastitis treatment.

The purpose of this study was to evaluate the effect of the nisin-based pharmaceutical formulation used in the treatment plan for cows suffering from subclinical mastitis on the milk microbiota. For this purpose the following tasks were set: to study the microbiota composition of milk from high yielding cows with subclinical mastitis; to analyze the comparative AMR profiles of microorganisms isolated from milk of subclinically mastitic cows; to study the effect of the nisin-based formulation used in the treatment regimen of cows with subclinical mastitis on the milk microbiota composition.

MATERIALS AND METHODS

Objects of the study: high yielding cows with subclinical mastitis, microorganisms isolated from milk, a nisin-based formulation.

The effect of the nisin-based formulation, used in the treatment regimen of cows with subclinical mastitis, on the milk microbiota composition was studied in 35 high yielding cows with a milk yield of more than 8,000 kg per year, kept at the nucleus farm in the Polevsky Raion of the Sverdlovsk Oblast. According to the treatment regimen of subclinical mastitis, the animals received 10 mL of a new pharmaceutical formulation intra-cisternally into the affected quarter daily for five days.

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).

During the study in 2023, the following isolates were recovered: *Staphylococcus aureus* (*n* = 32), *Streptococcus dysgalactiae* (*n* = 23), *Staphylococcus haemolyticus* (*n* = 20), *Enterococcus faecium* (*n* = 19), *Aerococcus viridans* (*n* = 17), *Staphylococcus epidermidis* (*n* = 13), coliform bacteria of *Escherichia* and *Enterobacter* genera (*n* = 8), *Corynebacterium pseudotuberculosis* (*n* = 5), *Mucor* (*n* = 4) and *Penicillium* spp. (*n* = 3) fungi.

A previously developed formulation containing nisin and water-based excipients was used with the following weight ratio percentage: nisin A – 0.3; silicon glycerolates

in a 6-mole excess of glycerol $Si(C_3H_7O_3)_4 \times 6C_3H_8O_3$ – 3.0; boron bisglycerolates $H[B(C_3H_6O_3)_2]$ – 2.0; glycerol – 10.0; distilled water – up to 100 [25, 26].

Test methods. The morphology of the recovered isolates was studied by seeding on Hiss growth media with sugars ("motley row"), was referenced by Bergey's Manual of Determinative Bacteriology [27], and the Guide to Clinically Significant Fungi [28], and then studied by MALDI-TOF mass spectrometry (Matrix-assisted laser desorption ionization time-of-flight mass spectrometry) using VITEK® MS (bioMérieux, France). For bacteriological and mycological testing, the milk samples were seeded on liquid and solid nutrient agars: meat peptone, *Streptococcus* Selective Agar, *Enterococcus* Selective Agar, Endo agar, *Staphylococcus* Selective Agar No. 10, Czapek medium, Sabouraud Dextrose Liquid Medium, bismuth sulfite agar, cetrimide agar, Levin medium, Ploskirev medium (State Research Center for Applied Microbiology and Biotechnology, Russia), 5% sheep blood agar (based on Columbia agar; Bio-Rad, France), defibrinated sheep blood (E&O Laboratories Ltd, Scotland), Salt Egg Yolk Agar Base (nutrient agar for microorganism culture GRM-agar, State Research Center for Applied Microbiology and Biotechnology, Russia), Uri*Select*4 nonselective chromogenic agar (Bio-Rad Laboratories, Inc., USA) and Sabouraud 2% Glucose Chloramphenicol Agar (SIFIN diagnostics GmbH, Germany).

The resistance of recovered isolates to 34 antimicrobial drugs from 15 groups (tetracyclines, penicillins, carbapenems, macrolides, lincosamides, ansamycins, amphenicols, I, II, III generation aminoglycosides, I, II, III generation cephalosporins, II, III generation fluoroquinolones) was evaluated by the disc diffusion test [29]. Commercially available discs were used (Scientific Research Center of Pharmacotherapy, Russia). The results were interpreted taking into account the recommendations of the European Committee on Antimicrobial Susceptibility Testing (EUCAST).

Statistical data were processed using AMRcloud, Microsoft Excel 2007 and Statistica 6.0. softwares.

RESULTS AND DISCUSSION

The studies were performed in 2023–2024 in the Department of Reproductive Biology and Neonatology, the Laboratory of Microbiological and Molecular Genetic Research of the Ural Federal Agrarian Research Center, Ural Branch of the Russian Academy of Sciences, in the laboratory of the medical center "Quality Med" LLC (Ekaterinburg) funded by the grant of the Russian Science Foundation No. 22-76-00009.

144 microorganisms were isolated from 70 milk samples from cows with subclinical mastitis, among them (Fig. 1): *S. aureus* (22.2%), *S. dysgalactiae* (16.0%), *S. haemolyticus* (13.9%), *E. faecium* (13.2%), *A. viridians* (11.8%), *S. epidermidis* (9.0%), coliforms of *Escherichia* and *Enterobacter* species (5.6%), *C. pseudotuberculosis* (3.5%), as well as *Mucor* (2.8%) and *Penicillium* spp. (2.1%) fungi.

In the present study, 81.4% of cows with subclinical mastitis were co-infected, among them 21.4% were co-infected with two pathogens, 28.6 and 17.1% of cows were infected with three and four pathogens. A complex microbiome consisting of five microorganisms was isolated in 14.3% of the samples.

The proportion of *S. aureus* isolates resistant to I, II and III generation cephalosporins was 53.1, 46.8 and 37.5%, respectively; to macrolides (erythromycin, clarithromycin) – 34.4%. Resistance to tetracyclines and penicillins was found in 31.3 and 28.1% of isolates, respectively. The minimum percentage of resistant *S. aureus* strains was reported for the following groups of antibiotics: II generation fluoroquinolones (12.5%), III generation fluoroquinolones (9.4%), carbapenems (6.3%). Intermediate resistance was established in 25.0% of isolates to amikacin (III generation aminoglycoside antimicrobial drug).

52.6% of *S. dysgalactiae* isolated strains showed resistance to tetracyclines (tigecycline, doxycycline). Non-sensitivity to III generation cephalosporins was established in 42.1% of isolates. Comparatively lower resistance was reported against II generation aminoglycosides (31.6%). 57.9% of *S. dysgalactiae* isolates demonstrated intermediate resistance to II generation cephalosporins (cefuroxim, cefoxitin).

Monitoring of antimicrobial resistance of *S. haemolyticus* isolates recovered from milk showed their resistance to macrolides (erythromycin, clarithromycin) in 33.3% of cases. A few isolates showed resistance to III generation cephalosporins (cefixime, cefoperazone, ceftriaxone) and ansamycins (rifampicin) – 13.3 and 6.7%, respectively. Intermediate sensitivity was revealed in 6.7% of the isolates to tetracyclines (doxycycline).

Resistance to penicillins, I generation aminoglycosides and III generation cephalosporins (42.1, 36.8 and 26.3%,

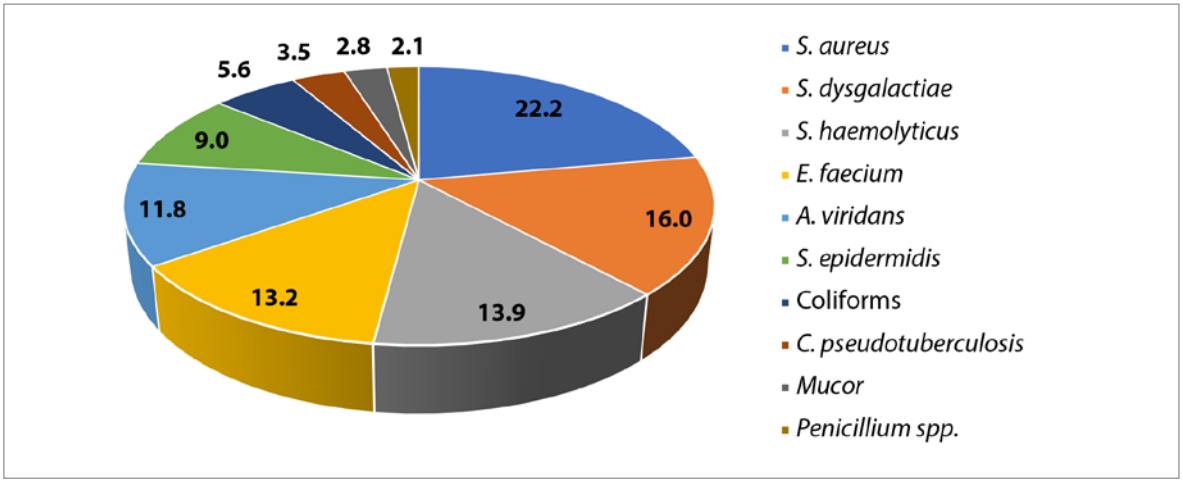

Fig. 1. Composition of the milk microbiota from cows with subclinical mastitis (n = 144)

Table 1
The structure of the microorganism population isolated from milk of cows with subclinical mastitis after using the new nisin-based formulation (*n* = 35)

Microorganism	Start of the experiment		After treatment course (Day 5)		Day 14 (from the beginning)	
	n	%	*n*	%	*n*	%
Monocultures						
S. aureus	8	22.9	4	11.4	3	8.6
S. dysgalactiae	4	11.4	2	5.7	–	–
A. viridans	2	5.7	1	2.9	–	–
S. epidermidis	1	2.9	–	–	–	–
C. pseudotuberculosis	1	2.9	–	–	–	–
S. haemolyticus	1	2.9	–	–	–	–
Coliforms	–	–	–	–	1	2.8
Associations						
S. aureus + coliforms + *E. faecium*	4	11.4	2	5.7	–	–
S. aureus + coliforms + *Streptococcus* spp. + *Penicillium* spp.	3	8.6	–	–	–	–
S. aureus + coliforms + *Streptococcus* spp. + *E. faecium*	3	8.6	–	–	–	–
S. aureus + coliforms + *Mucor*	2	5.7	–	–	–	–
S. aureus + coliforms + *Streptococcus* spp. + *Mucor*	2	5.7	–	–	–	–
S. aureus + coliforms + *Streptococcus* spp. + *E. faecalis* + *Mucor*	2	5.7	–	–	–	–
S. aureus + coliforms + *E. faecium* + *Streptococcus* spp.	1	2.9	–	–	–	–
S. aureus + *Streptococcus* spp. + *Mucor*	1	2.9	–	–	–	–
S. aureus + coliforms	–	–	1	2.9	–	–
E. faecalis + coliforms	–	–	1	2.9	–	–
Total	35	100	11	31.4	4	11.4

respectively) was demonstrated by *E. faecium* isolates. Intermediate sensitivity was found in 21.1% of the isolates to doxycycline from the tetracycline group.

The AMR profile of *A. viridans* isolates recovered from milk demonstrated the highest resistance to penicillins (ampicillin, amoxicillin, penicillin) – 35.3% and I generation aminoglycosides (kanamycin) – 23.5%. Intermediate susceptibility was revealed in 29.4% of the studied isolates to the tetracyclines (tetracycline, doxycycline).

38.5% of *S. epidermidis* isolates showed resistance to tetracyclines (tetracycline, doxycycline). Minimal resistance (15.4%) was reported against II generation aminoglycosides (gentamicin). 23.1% of the isolates demonstrated intermediate resistance to III generation fluoroquinolones (levofloxacin).

Isolated coliform bacteria had the greatest resistance (62.5%) to penicillins. Resistance to the ansamycins (rifampicin) was found in 37.5% of coliform isolates. Intermediate resistance to penicillins and II generation cephalosporins (cefuroxim, cefoxitin) was reported in 25.0 and 12.5% of coliform bacteria, respectively.

Corynebacterium pseudotuberculosis isolates were found to be equally resistant to penicillin and tetracycline antimicrobials (20.0%). Intermediate sensitivity was observed in 40.0% of the isolates to II generation cephalosporins (cefuroxim).

The obtained AMR profile of the milk microbiota from subclinically mastitic cows revealed the presence of multidrug-resistant coliform bacteria, *S. dysgalactiae*, *A. viridans*, *S. aureus* isolates. 62.5 and 47.1% of the tested coliform and *A. viridans* isolates respectively, had resistance to two groups of antimicrobials. Resistance of *S. dysgalactiae* to three antimicrobial groups was found in 43.5% of the isolated strains, to four groups in 26.1% isolates. 62.5% of the tested *S. aureus* isolates were resistant to four groups of antimicrobials, 46.9% of them were resistant to five groups and resistance to more than six groups of antimicrobials was reported in 15.6% of isolates.

The study showed that milk from subclinically mastitic cows has a complex microbiome, and the isolated microbiota are highly resistant to major antimicrobials used to treat mastitis. In this connection, a new pharmaceutical formulation based on bacteriocin nisin was included in the subclinical mastitis treatment regimen.

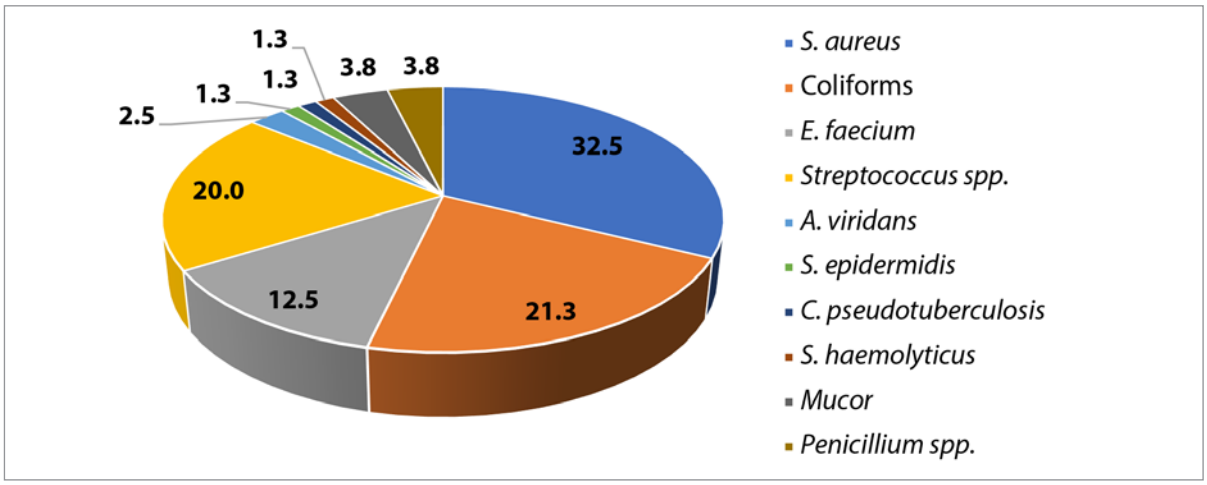

Fig. 2. The structure of microbial population isolated from milk samples from cows with subclinical mastitis at the beginning of the experiment (n = 80)

In milk samples from cows with subclinical mastitis, before using the nisin-based formulation, the isolated microorganisms were found both as a monoculture (48.6%) and as bacteria and fungi associations (51.4%). *S. aureus* (22.9%), *S. dysgalactiae* (11.4%), *A. viridans* (5.7%), *S. epidermidis* (2.9%), *C. pseudotuberculosis* (2.9%), *S. haemolyticus* (2.9%) were recovered as monocultures.

In the structure of bacterial associations, 11.4% of the samples were represented by *S. aureus* + coliforms + *E. faecium*; three-component associations included *S. aureus* + coliforms + *Mucor* (5.7%), *S. aureus* + *Streptococcus* spp. + *Mucor* (2.9%). Moreover, four-component associations were most often isolated from milk samples: *S. aureus* + coliforms + *Streptococcus* spp. + *Penicillium* spp. (8.6%), *S. aureus* + coliforms + *Streptococcus* spp. + *E. faecium* (8.6%), *S. aureus* + coliforms + *Streptococcus* spp. + *Mucor* (5.7%), *S. aureus* + coliforms + *E. faecium* + *Streptococcus* spp. (2.9%). The composition of the five-component associations was represented by *S. aureus* + coliforms + *Streptococcus* spp. + *E. faecalis* + *Mucor* (5.7%). The results are given in Table 1.

Eighty microorganisms in total were isolated from 35 milk samples at the beginning of the experiment, among them 74 bacteria and 6 fungi species (Fig. 2).

Herewith, the number of microbial cells in each sample was different. At the beginning of the experiment, 28.4% of *S. aureus* isolates were etiologically significant for the inflammation development in the mammary gland; the number of colony-forming units per 1 mL of the tested sample equal to 10^3, 10^6 and 10^7 CFU/mL was found in 9.5; 8.1 and 10.8% of the isolates, respectively. All 16 isolated *Streptococcus* spp. cultures (21.6%) were detected in the amount of 10^3 CFU/mL; 13.5% of coliform isolates, which can cause mastitis in animals, were in the amount of 10^5 CFU/mL. 10^3 and 10^5 CFU/mL values were found in 2.7 and 8.1% of *E. faecium* isolates, respectively. *S. epidermidis*, *C. pseudotuberculosis*, and *S. haemolyticus* were detected in the amount of 10^3 CFU/mL in 1.4% of cases. *A. viridans* were detected in a titer of 10^2 CFU/mL and from the beginning of the experiment were not an etiologically significant microorganism for mastitis development in cows (Table 2).

After the treatment course of animals with subclinical mastitis using the new nisin-based formulation, no microflora growth was observed in 68.6% of the samples (Table 1). The microbiota isolated from 11 samples of milk was a monoculture in 20.0% of cases represented by *S. aureus* (11.4%), *S. dysgalactiae* (5.7%), *A. viridans* (2.9%). In other samples, microorganism associations were detected: *S. aureus* + coliforms + *E. faecium* (5.7%); *S. aureus* + coliforms (2.9%); *E. faecalis* + coliforms (2.9%). 10^3 and 10^6 CFU/mL of *S. aureus* microbial cells were revealed in an equal number of isolates (1.4%). In 6.8% of cases, *S. aureus* isolated in a diagnostic titer, were not etiologically significant (10^2 CFU/mL), as well as coliforms, *E. faecium*, *A. viridans*, detected at the level of 10^1–10^2 CFU/mL. In one sample, *E. faecium* was detected in the amount of 10^3 CFU/mL, which accounted for 1.4% in the total structure of isolated microorganisms.

A microbiological testing of milk performed on Day 14 from the beginning of the treatment course showed an increase in the number of microbiota-free samples to 88.6%. In the tested samples, the milk microbiota was represented as a monoculture, where *S. aureus* and coliforms accounted for 8.6 and 2.8%, respectively (Table 1). 1.4% of *S. aureus* isolates were revealed in the amount of 10^3 CFU/mL. Coliforms and *S. aureus* isolated in 1.4% (10^1 CFU/mL) and 2.7% (10^2 CFU/mL) of cases respectively, were not etiologically significant in the diagnostic titer (Table 2).

Table 2
Number of bacteria, isolated from cow milk (n = 74)

Bacteria	CFU/mL	Start of the experiment		After treatment course (Day 5)		Day 14 (from the beginning of treatment)	
		n	%	*n*	%	*n*	%
S. aureus	10^2	5	6.8	5	6.8	2	2.7
	10^3	7	9.5	1	1.4	1	1.4
	10^6	6	8.1	1	1.4	–	–
	10^7	8	10.8	–	–	–	–
Coliforms	10^1	3	4.1	2	2.7	1	1.4
	10^2	4	5.4	1	1.4	–	–
	10^5	10	13.5	–	–	–	–
E. faecium	10^2	2	2.7	2	2.7	–	–
	10^3	2	2.7	1	1.4	–	–
	10^5	6	8.1	–	–	–	–
Streptococcus spp.	10^3	16	21.6	2	2.7	–	–
A. viridans	10^2	2	2.7	1	1.4	–	–
S. epidermidis	10^3	1	1.4	–	–	–	–
C. pseudotuberculosis	10^3	1	1.4	–	–	–	–
S. haemolyticus	10^3	1	1.4	–	–	–	–

In the last decade, intensive studies look at the potential of bacteriocins as next-generation therapeutics against drug-resistant bacteria [30, 31, 32, 33]. Bacteriocins from lactic acid bacteria are being tested as controlling agents for bacterial and viral infections; they can inhibit biofilm synthesis [33, 34, 35]. In a number of experiments, high antimicrobial activity of bacteriocin nisin was established against several species of staphylococci, including *Staphylococcus saprophyticus, S. aureus*, *S. epidermidis*, *S. haemolyticus* [36, 37, 38], including multidrug resistant and methicillin-resistant *S. aureus* [39]. There are studies on clinical isolates of *S. agalactiae* that have demonstrated different susceptibility to nisin [40]. Pérez-Ibarreche M. et al. [41] described the results of using nisin to effectively control biofilm of *S. uberis* strains that cause mastitis in cows. The use of nisin, which has antimicrobial activity against major mastitis-causing pathogens, could offer a potential alternative to antibiotics [36, 42, 43]. The data of our study confirm that it is feasible to include nisin into mastitis treatment regimens. During the experiment, it was found that the mastitis-causing pathogens isolated from milk, such as *S. aureus*, coliforms, *E. faecium*, *Streptococcus* spp., *A. viridans*, *S. epidermidis*, *C. pseudotuberculosis*, *S. haemolyticus*, are susceptible to the nisin-based formulation. Since the discovery of bacteriocins, researchers have mainly focused on testing their antimicrobial activity *in vitro*. However,

for the use of bacteriocins as antimicrobial drugs, it is necessary to study their clinical efficacy [44]. The effect of the nisin-based formulation on microbiota of milk from high yielding cows proved its effectiveness for treating subclinical mastitis: for example, in 88.6% of the samples, no microorganism growth was observed.

CONCLUSION

The study revealed the milk microbiota composition of high yielding cows with subclinical mastitis. It was found that in 81.4% of cases the disease occurs as a co-infection, among them two pathogens were isolated together in 21.4% of cases and three pathogens were isolated in parallel in 28.6% of cases. *S. aureus* (22.2%) and *S. dysgalactiae* (16.0%) were the most frequent isolated species.

A comparative analysis of the AMR of isolates recovered from milk of cows with subclinical mastitis showed the presence of multi-drug resistant strains of coliform bacteria, *S. dysgalactiae*, *A. viridans*, *S. aureus*. 62.5 and 47.1% of the tested coliforms and *A. viridans* isolates respectively, had resistance to two groups of antimicrobials. Resistance of *S. dysgalactiae* to three antimicrobial groups was found in 43.5% of the isolated strains, to four groups in 26.1% isolates. 62.5% of the tested *S. aureus* isolates were resistant to four groups of antimicrobials; 46.9% of them were resistant to five groups and resistance to more than six groups of antimicrobials was reported in 15.6% of isolates.

The study of the effect of the nisin-based formulation on microbiota of milk from cows with clinical mastitis revealed that after treatment 88.6% of the milk samples showed no microorganism growth. The milk microbiota in 8.6% of cases was represented by *S. aureus*, 2.8% were coliform bacteria. Herewith, in 1.4 and 2.7% of the samples, coliforms and *S. aureus* were detected in diagnostic titers equal to 10^1 and 10^2 CFU/mL, respectively, therefore they were not etiologically significant microorganisms for mastitis development.

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Dynamics of *Nakaseomyces glabratus* biofilm formation

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ABSTRACT

Formation of biofilms of microorganisms, including those of *Nakaseomyces glabratus*, is responsible for the development of local and systemic pathologies in humans and animals. The system of gene expression coordination (quorum sensing) in the representation of signaling molecules allows regulation of the amount and composition of biofilm populations thus expanding the adaptive capacity of microorganisms. In the presence of gingivitis and odontolithiasis clinical signs in dogs, excessive growth of gram-positive yeast microorganisms is a differential sign of the decreased resistance of the digestive system mucous membranes to colonization. Examination of the densitometric and morphometric parameters revealed general patterns of biofilm formation, regardless of the source of *Nakaseomyces glabratus* isolates. Depending on the time of cultivation of the microorganisms, a gradual increase in the optic density absolute values was established. Intercellular communications were achieved by coaggregation of the heteromorphic structures, which formed clusters with rounded liquid-containing formations detected among them. The population immobilization of the architectonics of the mature three-dimensional biofilm, as consistent with cultivation conditions, was accompanied by the differentiation of numerous cells of different sizes and shapes depending on the stage of the cell cycle. Results of the examination of the general patterns of the heterogeneous micromycete population development are promising for expanding the boundaries of knowledge of the adaptation mechanisms of ubiquitous microorganisms to long-term *in vivo* and *in vitro* persistence. Methods for studying morphometric and densitometric indicators avoiding interfering into the natural biofilm architectonics are recommended to optimize the long-term and retrospective mycological studies, as well as to develop effective mycosis treatment and prevention regimens.

Keywords: biofilms, microfungi, *Nakaseomyces glabratus*, optic density, microscopy, differential signs

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Динамика развития биопленок грибов *Nakaseomyces glabratus*

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РЕЗЮМЕ

Формирование биопленок микроорганизмов, в том числе и *Nakaseomyces glabratus*, обусловливает развитие локальных и системных патологий человека и животных. Система координации экспрессии генов (quorum sensing) при репрезентации сигнальных молекул позволяет регулировать количество и состав популяций биопленок, что расширяет адаптивный потенциал микроорганизмов. При наличии клинических признаков гингивита и одонтолитиаза у собак избыточный рост грамположительных дрожжевой формы микроорганизмов является дифференциальным признаком снижения колонизационной резистентности слизистой оболочки пищеварительной системы. Исследование денситометрических и морфометрических показателей выявило общие закономерности развития биопленок, независимо от источника выделения изолятов *Nakaseomyces glabratus*. В зависимости от времени культивирования микроорганизмов установили постепенное увеличение значений абсолютных величин оптической плотности. Реализация межклеточных коммуникаций достигалась коагрегацией гетероморфных структур, формирующих кластеры, между которыми выявлялись округлые образования, содержащие жидкость. Популяционная иммобилизация архитектоники зрелой трехмерной биопленки, в соответствии с условиями культивирования, сопровождалась дифференциацией многочисленных клеток разных размеров и форм в зависимости от стадии клеточного цикла. Результаты исследований общих закономерностей развития гетерогенной популяции микромицетов представляют перспективность для расширения границ познания механизмов адаптации убиквитарных микроорганизмов к длительной персистенции *in vivo* и *in vitro*. Способы изучения морфометрических и денситометрических

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показателей биопленок без нарушения естественной архитектоники рекомендуются для оптимизации микологических исследований, являющихся длительными и ретроспективными, а также разработки эффективных схем лечения и профилактики микозов.

Ключевые слова: биопленки, микроскопические грибы, *Nakaseomyces glabratus*, оптическая плотность, микроскопия, дифференциальные признаки

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INTRODUCTION

Nakaseomyces glabratus microfungi (formerly *Candida glabrata*) are recognized as one of the priority pathogens for research that cause development of nosocomial infections with high epidemiological mortality rates [1]. With the development of septicemia, endocarditis, pyelonephritis, bronchopneumonia, catheter-, prosthesis-associated pathologies, *N. glabratus* isolates are significantly often characterized by multidrug resistance [2, 3, 4].

The tendency of a statistically significant increase in the etiological significance of *N. glabratus* has been established with the development of opportunistic endogenous infection of animals against the antibiotic therapy [5, 6, 7, 8, 9, 10].

With the development of micromycete overgrowth syndrome, the pathogenic potential is realized by increasing the biofilm biomass, which are a heterogeneous association of microorganisms aggregated by the extracellular matrix [11, 12, 13].

Gene expression coordination system (quorum sensing), when representing signaling molecules, allows regulation of the number and composition of biofilm populations thus expanding adaptive potential of the microorganisms [12, 14, 15].

Transcriptional control of adhesion, invasion, and synthesis of polymeric substances allows the multicellular population to realize virulent properties in interaction with immunocompetent cells, provides protection against phagocytosis and effects of chemotherapeutic drugs and disinfectants [16, 17, 18].

Initiation, development and outcome of superficial, deep and systemic mycoses with excessive growth and increase in pathogenic potential of ubiquitous microorganisms are attributed to hyperaggregation, presence of dissociative variants and dispersion of heterogeneous biofilms. The investigation of the ways to indicate biofilms, including when exposed to chemotherapeutic drugs and disinfectants, in the future will allow the development of fungicidal medicinal products aimed to block the synthesis or destruction of the biofilm extracellular matrix.

To improve the procedures of mycological research and development of preventive antiepidemic measures, the priority area of scientific investigations is to expand the boundaries of knowledge of the mechanisms of the multi-stage biofilm formation process.

The aim of the work was to study the morphometric and densitometric parameters of *N. glabratus* isolates recovered and identified during the progress of gingivitis and odontolithiasis in dogs.

MATERIALS AND METHODS

Strains. N. glabratus isolates were used in the experiments, which were recovered from the swabs and scrapings of the oral mucosa of dogs demonstrating gingivitis and odontolithiasis clinical signs. Reference strain ATCC 66032 was tested as control [19].

Nutrient media. Meat-extract broth (HiMedia Laboratories Pvt. Ltd., India), bovine blood serum (Microgen, Russia); rice extract agar (API-System R.A.T., France); Sabouraud glucose agar with penicillin and streptomycin (100 IU/L); Columbia agar; chromogenic agar (BioMedia, Russia).

Test-kits. HiCandida™ Identification Kit (HiMedia Laboratories Pvt. Ltd., India) was used to identify the microorganisms.

Indication and identification of microorganisms. For microscopy, fingerprints were prepared from the swabs and scrapings and Gram-stained.

Medium quality control – sterility test at (36 ± 1) °C for 48 hours.

To indicate hyphal germ tubes, the day-old cultures of the microorganisms were cultured in meat-extract broth supplemented with 1.0 mL of blood serum at (35 ± 2) °C for 5 hours and microscopic examination of the methylene blue-stained specimens was performed.

Chlamydospore presence was tested on native microorganism culture specimens grown on rice agar at (25 ± 2) °C for 24 hours.

When adding cycloheximide (0.5 g/L of the medium) to Sabouraud agar, the growth of microorganisms at (25 ± 2) °C during 72 hours was recorded.

The microorganisms were indicated and identified with due consideration of the typical growth properties of the microorganisms and using generally accepted methods [20, 21].

To record the biochemical properties, the day-old cultures of the microorganisms (optical density OD = 0.5; wavelength 620 nm) were added into the wells of HiCandida™ Identification Kit panel and cultivated at (22.5 ± 2) °C for 48 hours. The microorganisms were identified in accordance with the identification tables and codes of the specified test-kit.

Investigation of the biofilm formation dynamics. To record the biofilm formation dynamics, the microorganisms were cultivated under static conditions at (35 ± 2) °C for 18, 24 and 48 hours.

To assess the densitometric parameters, upon the specified cultivation period completion, the fluid was removed and the sediment was washed three times with 200.0 µL of the phosphate buffer solution (pH 7.2). The specimens were fixed with 150.0 µL of 96° ethanol for 15 minutes. The specimens were further dried at (35 ± 2) °C for 20 minutes, 0.5% crystalline violet solution was added and transferred to the thermostat at (35 ± 2) °C for 5 minutes. The contents of the wells were removed, the wells were washed three times with 200.0 µL of the phosphate buffer solution (pH 7.2) and dried. The stain was eluted with 200.0 µL of 96° ethanol for 30 min [13].

The OD of the tested specimens was determined by the degree of crystalline violet binding (HiMedia Laboratories Pvt. Ltd., India) using a photometric analyzer Immuno-Chem-2100 (High Technology Inc., USA) at wavelength 580 nm (OD_{580}).

For morphometric tests, the slides were placed in Petri dishes and 100.0 mL of 18-hour cultured microorganism suspension were added at 10^5 CFU/mL. After a predetermined time of the microorganisms' cultivation, the specimens were fixed three times, sequentially immersed in 96° ethanol for 10 minutes and air-dried for 10 minutes. Then the specimens were stained with gentian violet solution supplied with the Gram staining kit (BioVitrum, Russia).

During representative sampling of significant frequency of occurrence (≥ 90.0% of the field of Carl ZEISS Axio Lab.A1 optical microscope, Germany) microphotography was made using ADF PRO 08 digital camera (China) with matrix resolution of 8 megapixels (4K).

The resulted data were processed by statistical analysis method using the Student's criterion, the results were considered reliable at $p < 0.05$ [18].

RESULTS AND DISCUSSION

Indication and identification of microorganisms. With gingivitis and odontolithiasis clinical signs in dogs, intense yellow-gray deposits tightly attached to the mucous membrane were observed. Bright red ulcers were generally detected when these deposits were removed.

Optical microscopy of fingerprints of oral mucosa scrapings revealed a large number of gram-positive yeast-shaped microorganisms of (1.1–2.1 × 3.1–4.0) µm in size.

After 48 hours at (35 ± 2) °C, the microorganisms formed shiny white colonies on Sabouraud agar.

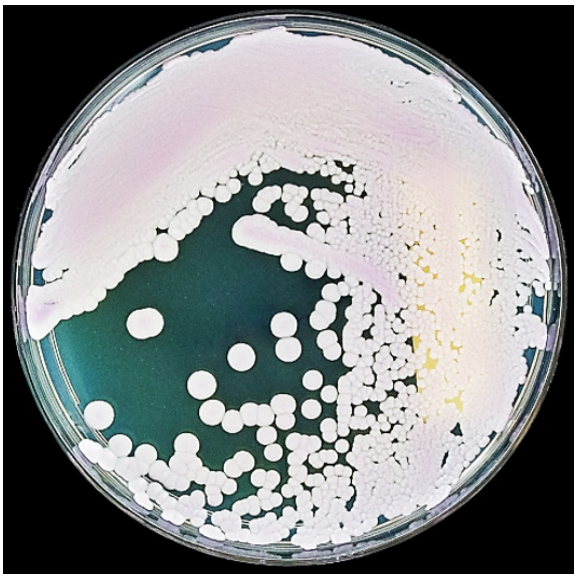

Fig. 1. Features of N. glabratus growth on chromogenic agar at (35 ± 2) °C for 48 hours

Presence of chromogenic substrates in the indicator medium allowed differentiation of *N. glabratus* colonies of pink color with a slightly purple hue (Fig. 1).

Nakaseomyces glabratus microfungi fermented maltose, trehalose; they did not ferment urease, melibiose, lactose, sucrose, galactose and xylose. The microorganisms did not have any urease activity.

Microscopic examination of the specimens of the microorganisms cultured in 1 mL serum supplemented-meat-extract broth at (35 ± 2) °C for 5 hours did not reveal formation of hyphal germ tubes; therefore, the test result was negative.

In microorganism cultures grown on rice agar at (25 ± 2) °C for 24 hours, chlamydospores were not detected; therefore, the test result was negative.

When incubating the culture on cycloheximide-containing Sabouraud agar (0.5 g/L) at (25 ± 2) °C for 72 hours, no microorganisms' growth was observed; therefore, the test result was negative.

When recording the microorganisms' tolerance to temperature (35 ± 2), (42 ± 2), (45 ± 2) °C for 24 hours, typical turbidity of the liquid Sabouraud medium, presence of a slight sediment and gray thin film on the surface of the medium were detected; therefore, the test result was positive.

The reference strain and isolates, regardless of the source of isolation, demonstrated properties typical of yeast-like fungi of *N. glabratus* species (Table).

Table
Differential features of *N. glabratus*

Microorganism cultures	Differential signs					
	Hyphal tubes	Chlamydospores	Cycloheximide	Temperature tolerance		
				(35 ± 2) °C	(42 ± 2) °C	(45 ± 2) °C
N. glabratus, ATCC 66032	–	–	–	+	+	+
N. glabratus, swabs from the dorsal surface of the tongue	–	–	–	+	+	+
N. glabratus, swab from the gums	–	–	–	+	+	+

"+" – presence of microbial growth; "–" – absence of microbial growth.

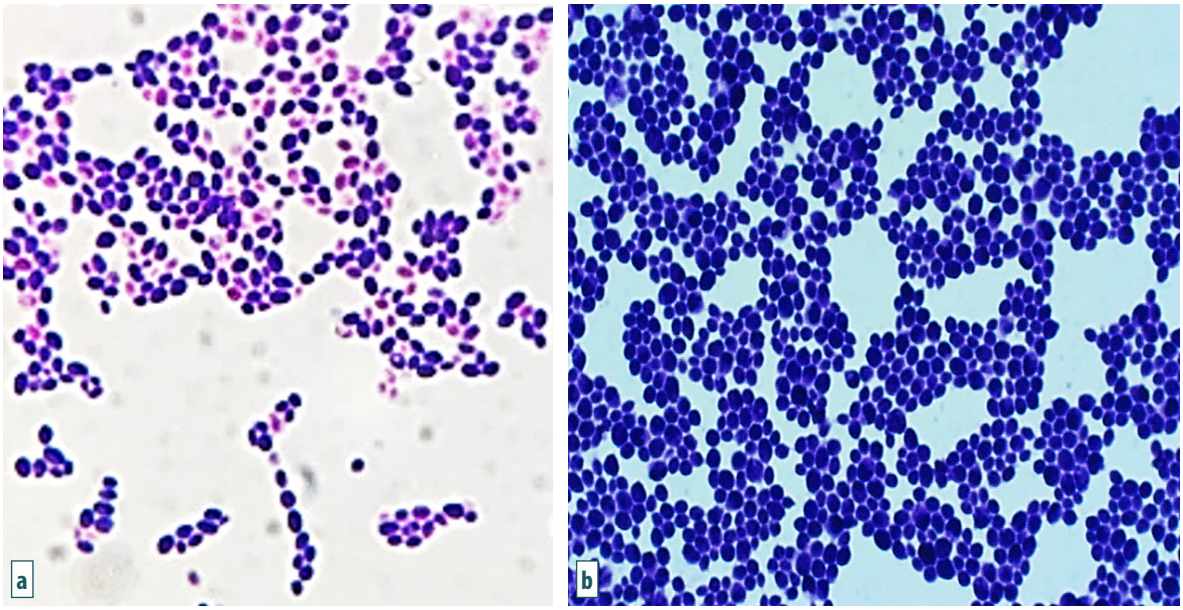

Fig. 2. Stages of N. glabratus biofilm formation at (35 ± 2) °C in beef-extract broth (yeast-like cells of species-typical shape and size aggregated by the extracellular matrix): a – in 18 hours; b – in 24 hours. Methylene blue staining, ocular lens 10×, objective lens 100×, immersion

Biofilm formation dynamics. The study of densitometric and morphometric parameters revealed common patterns of biofilm formation of *N. glabratus* isolates, regardless of the source of isolation.

Depending on the time of cultivation of the microorganisms, a gradual increase in the OD absolute values of the tested specimens was established: after 18 hours – from 0.218 ± 0.05 to 0.221 ± 0.08, the intensity of biofilm formation – ≥ 0.1; after 24 hours – from 0.289 ± 0.04 to 0.297 ± 0.09, the intensity of biofilm formation – ≥ 0.2; after 48 hours – from 0.331 ± 0.10 to 0.350 ± 0.08, the intensity of biofilm formation – ≥ 0.3.

During representative sampling according to the morphometric parameters of the significant frequency of occurrence (≥ 90.0% in the field of view of an optical microscope – yeast cells of shape and size typical for *N. glabratus* species were found aggregated by the extracellular matrix (Fig. 2).

Depending on the time of cultivation, such stages as adhesion, fixation, coagulation, microcolonies, and dispersion were revealed during the biofilm formation.

At the early stages of the formation, primary attachment due to planktonic forms sorption, i.e. adhesion of the microorganisms to the test substrate, was reported (in our studies – to the slide surface). This stage is considered reversible, that is, the attached cells can detach from the substrate and return to the planktonic form.

During the adhesion, the cell walls of the microorganisms produced exocellular molecules that ensured the cell fixation to the slide surface. The cells tightly fixed to the substrate contributed to the adhesion of the subsequent cells.

The population immobilization of the architectonics of the mature three-dimensional biofilm dependent on the cultivation conditions is mediated by the quorum

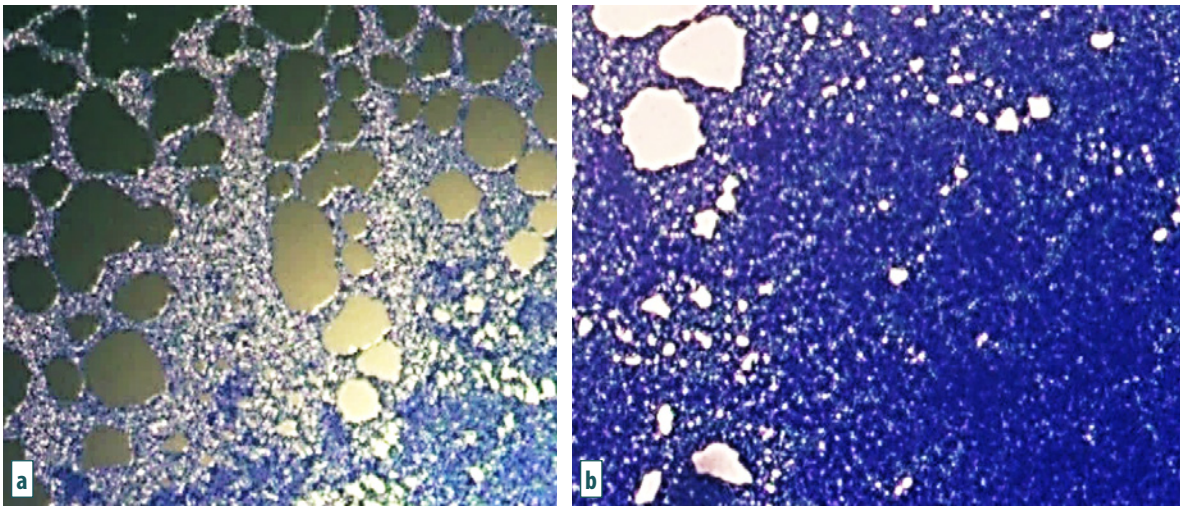

Fig. 3. Stages of N. glabratus biofilm formation at (35 ± 2) °C in beef-extract broth in 48 hours: intense cell proliferation is accompanied by the formation of net-like structures and extracellular matrix thickening. Gentian violet staining: a – ocular lens 10×, objective lens 5×; b – ocular lens 10×, objective lens 10×

sensing phenomenon. This is a special form of extracellular communication of the microorganisms due to the synthesis of numerous exocellular molecules, whose concentration is proportional to the cell density.

When increasing the cultivation time, intensive proliferation was accompanied by differentiation of yeast forms and formation of an extracellular matrix (Fig. 3).

The number of the attached dividing cells significantly increased and, accordingly, the growth of microcolonies was observed, which resemble colonies formed on dense nutrient media. With an increase in the number of microorganisms due to an increase in the synthesis of exocellular molecules, extracellular bonds were formed, and the population, called a mature biofilm, was immobilized.

When certain sizes of microcolonies were reached, dispersion of individual cells periodically occurred, which were after a while able to attach to the surface and form a new microcolony.

The general pattern of the regularity and compactness of the multicellular heteromorphic biofilm population was determined by the cell cycle stages and degree of development of the extracellular matrix. The cellular composition of the mature biofilm was represented by ovoid- or ellipsoid-shaped cells of typical size of (1.1–1.9) × (3.1–3.4) microns (Fig. 4).

The population immobilization of the architectonics of the mature three-dimensional *N. glabratus* biofilm, as consistent with cultivation conditions, was accompanied by the differentiation of numerous cells of different sizes and shapes depending on the cell cycle stage [12, 15, 18].

Results of the examination of the general patterns of the heterogeneous microorganism population development are promising for expanding the boundaries of knowledge of the adaptation mechanisms of ubiquitous microorganisms to long-term *in vivo* and *in vitro* persistence.

Methods for studying morphometric and densitometric indicators avoiding interfering into the natural biofilm architectonics are recommended to optimize the long-term and retrospective mycological studies, as well as to develop effective mycosis treatment and prevention regimens.

CONCLUSION

Microscopy of the fingerprints of the scrapings of the oral mucosa of dogs demonstrating gingivitis and odontolithiasis clinical signs revealed excessive growth of gram-positive yeast microorganisms. Depending on the time of cultivation of the microorganisms, a gradual increase in the OD absolute values was established. Extracellular communications were achieved by coaggregation of the heteromorphic structures forming clusters, among which rounded liquid-containing formations were detected. Regularity and compactness of the multicellular heteromorphic population of mature biofilm is determined by the cell cycle stages and degree of the extracellular matrix development.

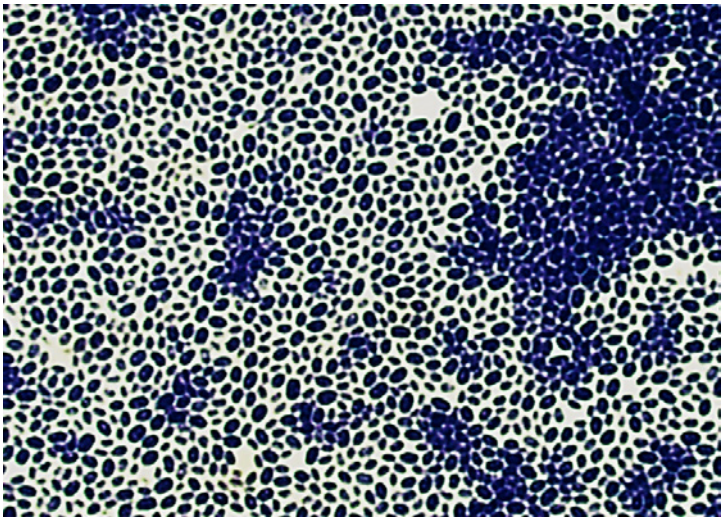

Fig. 4. Stage of N. glabratus biofilm formation at (35 ± 2) °C on beef-extract broth in 48 hours: regularity and compactness of multicellular heteromorphic population of the mature biofilm. Gentian violet staining, ocular lens 10×, objective lens 100×, immersion

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Characteristics of the intestinal tract microbiota in calves with various forms of acute catarrhal bronchopneumonia

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ABSTRACT

The intestinal barrier is one of the most important components that maintain gastrointestinal homeostasis, therefore changes in bacterial composition can lead to increased intestinal permeability and the development of intestinal translocation of opportunistic microorganisms, with the subsequent development or complication of various infectious diseases. A comparative description of the microbiota of the intestinal tract of calves with compensated, subcompensated and decompensated acute catarrhal bronchopneumonia of calves was carried out in the conditions of livestock farms of Vladimir and Moscow Oblasts. Calves aged 1–3 months with acute catarrhal bronchopneumonia ($n = 37$) were used for the study. The severity of the disease was assessed based on clinical and laboratory tests. The samples taken from clinically healthy animals ($n = 8$) were used as controls. It has been shown that in calves with compensated acute catarrhal bronchopneumonia, the qualitative and quantitative composition of the intestinal microbiome does not differ from clinically healthy animals. During the clinical manifestation of subcompensated and decompensated acute catarrhal bronchopneumonia in calves, a significant quantitative and qualitative shift in the microbiome occurs in the intestines, which indicates the occurrence of dysbiosis. We believe that this area is quite relevant and requires further scrupulous research.

Keywords: bronchopneumonia, intestinal dysbiosis, microbiota, biotope, intestinal translocation, calves

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Характеристика микробиоты кишечного тракта у телят с различными формами острой катаральной бронхопневмонии

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РЕЗЮМЕ

Кишечный барьер является одним из важнейших компонентов, поддерживающих гомеостаз в желудочно-кишечном тракте, поэтому изменения бактериального состава могут привести к повышенной проницаемости кишечника и кишечной транслокации условно-патогенных микроорганизмов с последующим развитием либо осложнением различных инфекционных заболеваний. Проведена сравнительная характеристика микробиоты кишечного тракта телят с компенсированной, субкомпенсированной и декомпенсированной острой катаральной бронхопневмонией в условиях животноводческих ферм Владимирской и Московской областей. Объектом исследования служили телята в возрасте 1–3 мес., больные острой катаральной бронхопневмонией ($n = 37$). Оценку степени тяжести заболевания осуществляли на основании проведенных клинико-лабораторных исследований. Контролем служил материал, отобранный от клинически здоровых животных ($n = 8$). Показано, что у телят при компенсированной острой катаральной бронхопневмонии

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качественный и количественный состав кишечного микробиома не отличается от клинически здоровых животных. При клинической манифестации субкомпенсированной и декомпенсированной острой катаральной бронхопневмонии у телят в кишечнике происходит существенный количественный и качественный сдвиг микробиома, что свидетельствует о возникновении дисбактериоза. Считаем, что данное направление достаточно актуально и требует дальнейших скрупулезных исследований.

Ключевые слова: бронхопневмония, дисбактериоз кишечника, микробиота, биотоп, кишечная транслокация, телята

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INTRODUCTION

The number of cattle in livestock complexes has recently increased due to dairy farming intensification. This, in turn, creates unfavorable conditions resulting in reduced animal resistance to various adverse environmental effects, including the negative impact of opportunistic microflora associations circulating in farm biogeocenoses [1, 2, 3]. Factor infections are quite common in cattle and cause significant economic losses in livestock. The most common factor infections include obstetric and gynecological diseases in cows, as well as pneumoenteritis in calves. To prevent these cattle infections, it is necessary to follow preventive measures, including regular vaccination, maintaining hygiene in animal pens, monitoring feeding and watering, as well as regular examination and treatment of diseased animals [4, 5, 6].

Respiratory diseases which are most often diagnosed in young animals are currently widespread in livestock farms among highly productive animals. At the same time, economic losses for the industry comprise animal mortality, drop in diseased or recovered animal productivity, retarded growth and development, and costs on treatment and prevention [7]. Bronchopneumonia of calves is registered in nearly all areas of our country and it ranks second after gastrointestinal diseases among all in-farm animal pathologies, reaching 20–30% [8]. The etiological factors of nonspecific bronchopneumonia of calves are a complex of causes: high density of animal housing, decreased resistance and immunological reactivity of newborn animals, exposure to adverse environmental factors, stress, feeding imbalance, as well as conditionally pathogenic microbiota of the respiratory tract, which under these conditions can acquire pathogenic properties [9, 10, 11].

Trillions of microorganisms living in the intestinal tract are important health regulators, therefore, qualitative and quantitative disorders in microbial biotopes of the intestine can cause or complicate various infectious diseases [12, 13, 14, 15]. Leaky gut syndrome is a condition characterized by increased intestinal permeability. Since the intestinal barrier is one of the most important structures supporting homeostasis in the gastrointestinal tract, loss of its integrity due to changes in bacterial composition, decreased levels of expression of dense compound proteins and increased concentrations of proinflammatory cytokines can lead to increased intestinal permeability with the subsequent development of gastrointestinal and non-gastrointestinal diseases. The translocation of microorganisms and their toxic metabolites outside the biotope being the gastrointestinal tract, is one of the consequences of the leaky gut syndrome [16, 17, 18].

Translocation of the intestinal microbiota is a process where microorganisms from the intestinal tract penetrate through its wall into the bloodstream and distribute in the body. This may play a significant role in the development of infectious diseases of various etiology, including respiratory ones. Thus, pathogenic microorganisms entering the bloodstream by intestinal translocation can cause a systemic inflammatory response in the body, thus deteriorating the course of infection. Also, bacteria penetrating through the intestinal wall may cause metastatic infections in various organs and tissues, leading to complications and a more severe course of the disease. In addition, intestinal translocation of microorganisms can facilitate bacteremia, which might result in sepsis and shock. The entry of infectious agents from the intestinal tract into the bloodstream may also cause recurrence of infection after completion of antibiotic therapy [19, 20, 21, 22].

In general, intestinal translocation of microorganisms plays an important role in the development and course of various infectious diseases, therefore, control of this process may be a key aspect in the treatment of such conditions. In this regard, the study of the role of intestinal translocation of microorganisms in acute catarrhal bronchopneumonia of calves of varying severity is an urgent issue requiring timely and competent solutions.

The novelty of the paper consists in the fact that it is for the first time that the condition of the rectal biotope in calves with acute catarrhal bronchopneumonia of varying severity was studied. It has been shown that, depending on the severity of the infectious process, significant qualitative and quantitative shifts occur in the setting of intestinal dysbiosis.

The aim of the work is to conduct a comparative analysis of the intestinal microbiota in calves with compensated, subcompensated and decompensated acute catarrhal bronchopneumonia on the livestock farms in the Vladimir and Moscow Oblasts.

MATERIALS AND METHODS

The research was supported by the grants of the Russian Science Foundation (project No. 24-26-00091, https://rscf.ru/project/24-26-00091) and conducted on the basis of livestock farms in the Vladimir and Moscow Oblasts with a total cattle population of 3,680 animals, including 1,690 cows.

Bacteriological tests were performed in the VETTEST veterinary laboratory (Moscow).

The study was aimed at 1–3 months-old calves with acute catarrhal bronchopneumonia (n = 37). The diagnosis was established based on comprehensive data, taking into account the medical history, clinical examination and microbiological tests. The animals that received medical treatment within 14 days prior to sampling were removed from the experiment. The severity of acute catarrhal bronchopneumonia of calves (1st degree – compensated; 2nd degree – subcompensated; 3rd degree – decompensated) was assessed on the basis of clinical and laboratory tests. The animals were divided into three groups based on the severity of bronchopneumonia: calves with the mild degree (compensated, n = 12), moderate degree (subcompensated, n = 14) and severe degree (decompensated, n = 11) of the disease severity. The samples collected from clinically healthy animals were used as controls (n = 8).

Fecal samples were collected from experimental calves in the morning hours and placed into sterile test tubes. For microbiological tests, the pathological samples were inoculated onto nutrient media using a Pasteur pipette. Sabouraud glucose agar was used for yeast-like fungi; peptone-salt medium, yolk-salt agar and meat-peptone agar (MPA) were used for staphylococci; Endo agar, Ploskirev medium, King medium and bismuth-sulphite agar – for enterobacteria; Blaurocca medium – for bifidobacteria; skimmed milk and MRS agar – for lactobacilli. The inoculations were then incubated in a thermostat at 37–38 °C for 24 hours, and if no growth was observed, the dishes were kept longer for up to 3 days. After testing the cultural and morphological properties, all species colonies were separately inoculated into test tubes and incubated at 37–38 °C for 24 hours. The resulting pure bacterial cultures were tested for mobility in crushed droplet preparations using phase contrast microscopy in a darkened field of view and subjected to identification.

For quantitative bacteriological test, 1.0 g of feces were collected and added into sterile tubes with sterile sodium chloride saline solution (9.0 cm^3). The contents of the first tube, considered as 10^{-1} dilution, was used for preparing further ten-fold dilutions up to 10^{-10}. Then, 0.1 cm^3 of the resulting mixture from each tube was inoculated into Petri dishes onto solid nutrient media (Endo, MPA, Sabouraud, Blaurocca, MRS, PSL, King, Ressel, bismuth-sulphite agar, yolk-salt agar).

The number of microorganisms (C) in 1.0 cm^3 of feces collected from calves with acute catarrhal bronchopneumonia was calculated using the formula below and expressed in logarithms with a base of 10:

$$C = (N/V) \times K,$$

where N is the mean number of colonies in one bacteriological dish; V is the volume of suspension applied during inoculation onto agar (cm^3); K is the multiplicity of dilution.

The morphology of bacteria was tested in smears stained according to Gram and Romanovsky – Giemsa. Further identification based on biochemical properties was carried out in accordance with Bergey's Manual of Determinative Bacteriology[1].

The obtained test results were processed statistically and presented in the tables. All calculations were performed using the STATISTICA 7.0 program (StatSoft, USA), while the normality of the distribution was preliminarily estimated using Shapiro – Wilk tests. In case of a normal distribution of quantitative variables, the Student's independent samples t-test was used to compare the two groups. The arithmetic mean (Mean), standard error (SE) and standard deviation (SD) were calculated. The reliability of the analyte difference between the indicators of the control and experimental groups was calculated using the Mann – Whitney method[2].

RESULTS AND DISCUSSION

During the epizootological examination, 37 calves (1–3 months of age) with acute catarrhal bronchopneumonia were identified. Based on clinical study results 12 animals were classified as having a mild, compensated disease stage (slight depression, subfebrile body temperature, shallow hard breathing, serous nasal discharge), 14 calves had a medium, subcompensated disease stage (depression, fever, increased pulse and respiration, dry wheezing and cough, abundant serous catarrhal discharge), and 11 calves had a severe, decompensated disease stage (pronounced depression and exhaustion, inappetence, decreased reaction to external stimuli, fever, increased pulse and respiration, painful dry cough, wheezing, foci of dulling percussion sound, abundant serous-catarrhal exudate of greenish color).

Microbiological studies of bronchoalveolar lavage samples collected from diseased animals have shown that the occurrence of bronchopneumonia in calves is due to a fairly wide range of conditionally pathogenic microflora. Thus, 115 bacteria of thirteen species classified into nine genera were isolated from the bronchial samples. At the same time, the following strains were isolated more often from bronchoalveolar lavage samples*: Staphylococcus aureus* – 18 (15.6%) cultures, *Mannheimia haemolytica* – 18 (15.6%) strains, *Escherichia coli* – 15 (13.1%) isolates, *Pasteurella multocida* – 11 (9.6%) cultures and *Klebsiella pneumonia* – 11 (9.6%) strains. *Staphylococcus intermedius* and *Proteus mirabilis* were isolated much less frequently – three (2.6%) cases per each species, respectively.

The quantity of microorganisms (lg) in 1 g of feces from calves with compensated, subcompensated and decompensated acute catarrhal bronchopneumonia is shown in the Table.

The analysis of the results showed that the qualitative and quantitative composition of the intestinal microbiome in calves with compensated acute catarrhal bronchopneumonia does not differ from that in clinically healthy animals.

The presented data demonstrate that significant quantitative and qualitative changes occur in the intestinal

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biotope of animals with a clinical manifestation of moderate severity of acute catarrhal bronchopneumonia, which indicate the development of dysbiosis. The fecal samples from calves with subcompensated bronchopneumonia demonstrated a reliable increase in *Enterobacter* spp. by 1.26 times ($p < 0.05$), *Citrobacter* spp. by 1.35 times ($p < 0.05$), *Klebsiella* spp. by 1.90 times ($p < 0.01$), *Proteus* spp. by 1.66 times ($p < 0.01$), *Pseudomonas* spp. by 2.58 times ($p < 0.001$), *Clostridium* spp. by 2.06 times ($p < 0.001$) and *Candida* spp. by 2.12 times ($p < 0.01$) when compared with the indicators for the control group animals. This was observed against the background of a highly reliable ($p < 0.001$) decrease in representatives of the genera *Lactobacillus* and *Bifidobacterium* from (10.42 ± 0.72) to (8.59 ± 0.76) lg and from (10.94 ± 0.73) to (9.06 ± 0.62) lg, by 17.56 and 17.18%, respectively, when compared with the indicators for clinically healthy calves.

The results shown in the Table also indicate that significant quantitative and qualitative dysbiotic shifts in the microbiota of the intestinal tract were observed in calves with the most severe decompensated degree of acute catarrhal bronchopneumonia. Thus, the testing of fecal samples from diseased animals showed a significant increase in the amount of representatives of the following genera: *Escherichia* by 1.21 times ($p < 0.001$), *Enterobacter* by 1.63 times ($p < 0.001$), *Citrobacter* by 1.83 times ($p < 0.001$), *Klebsiella* by 3.08 times ($p < 0.001$), *Proteus* by 1.90 times ($p < 0.001$), *Pseudomonas* by 3.57 times ($p < 0.001$), *Staphylococcus* by 1.24 times ($p < 0.05$), *Streptococcus* by 1.38 times ($p < 0.01$), *Bacillus* by 1.47 times ($p < 0.01$), *Clostridium* by 3.34 times ($p < 0.001$) and yeast fungi (genus *Candida*) by 2.82 times ($p < 0.001$), when compared with the indicators for the control group animals. The presented quantitative differences were recorded against the background of a highly reliable decrease in the amount of the following species in fecal samples of experimental animals: *Lactobacillus* spp. ($p < 0.001$) and *Bifidobacterium* spp. ($p < 0.001$) from (10.42 ± 0.72) to (6.51 ± 1.08) lg and from (10.94 ± 0.73) to (7.36 ± 0.81) lg, by 37.5 and 32.7%, respectively, as compared with the control group. That was the first time that we obtained these data.

The qualitative parameters of the intestinal microflora in calves with acute catarrhal bronchopneumonia of compensated, subcompensated and decompensated severity are shown in the Figure.

As we can see, the representatives of the genera *Klebsiella*, *Pseudomona* and *Clostridium* were not detected in the most mild, compensated forms of the disease, however, were isolated in the more severe course of the disease – subcompensated and decompensated degrees of severity. It can be assumed that the severity of bronchopneumonia may be associated with intestinal dysbiosis and the phenomenon of intestinal translocation of microorganisms from a natural, evolutionarily developed biotope to the focus of destruction – the lungs. Therefore, further studies should be aimed at determining the identity of the intestinal microflora with microorganisms isolated from lung tissue in the setting of bronchopneumonia.

Thus, the new criteria for assessing the severity of acute catarrhal bronchopneumonia in calves have been suggested. To this extent, when subcompensated and decompensated acute catarrhal bronchopneumonia is clinically manifested in calves, significant quantitative and qualitative microbiome disorders occur in the intestine, indicating dysbiosis. The development of intestinal dysbiosis may serve as a kind of trigger for the formation and progression of pathologies of the respiratory tract. The data obtained comply with the study results on intestinal microbiome in inflammatory processes of various localization [20, 23]. This confirms the important role of the intestinal microbiota and intestinal permeability (normal and increased) in the manifestation of many infectious diseases. However, the currently available data are selective and insufficient, their assessment is a matter of debate, and their clinical

Table
Composition and quantity (lg) of opportunistic microflora in 1 g of calf feces in various forms of acute catarrhal bronchopneumonia ($M \pm m$)

Microorganism genus	Control ($n = 8$)	Mild degree ($n = 12$)	Medium degree ($n = 14$)	Severe degree ($n = 11$)
Escherichia	7.07 ± 0.96	7.40 ± 0.77	7.69 ± 0.79	8.55 ± 0.61***
Enterobacter	2.65 ± 0.70	2.72 ± 0.77	3.35 ± 0.50*	4.31 ± 0.82***
Citrobacter	2.26 ± 0.75	2.34 ± 0.78	3.05 ± 0.84*	4.14 ± 0.70***
Klebsiella	0	0	1.90 ± 1.43**	3.08 ± 1.02***
Proteus	1.79 ± 0.51	2.01 ± 0.69	2.97 ± 0.85**	3.40 ± 0.76***
Pseudomonas	0	0	2.58 ± 0.72***	3.57 ± 0.78***
Staphylococcus	3.61 ± 0.78	3.50 ± 0.71	3.93 ± 0.75	4.47 ± 0.76*
Streptococcus	3.59 ± 0.75	3.26 ± 0.91	4.17 ± 0.65	4.95 ± 0.78**
Bacillus	2.74 ± 0.89	2.65 ± 0.85	3.13 ± 0.49	4.02 ± 0.97**
Clostridium	0	0	2.06 ± 1.13***	3.34 ± 0.67***
Lactobacillus	10.42 ± 0.72	10.27 ± 0.67	8.59 ± 0.76***	6.51 ± 1.08***
Bifidobacterium	10.94 ± 0.73	10.72 ± 0.84	9.06 ± 0.62***	7.36 ± 0.81***
Candida	1.29 ± 1.48	1.28 ± 1.27	2.74 ± 0.77**	3.64 ± 0.88***

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ as compared with the control group.

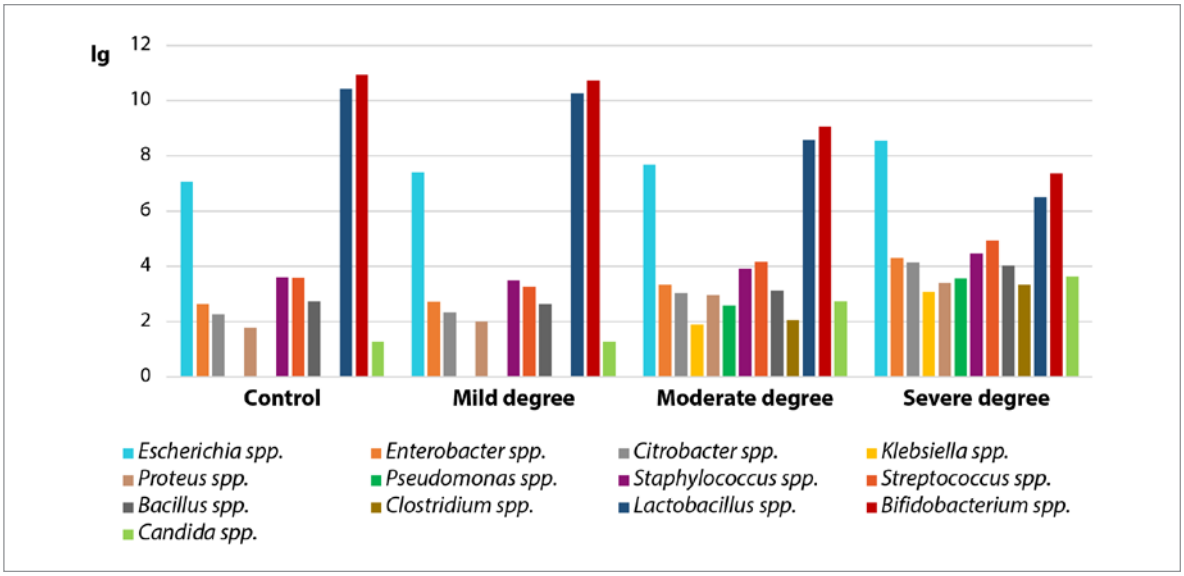

Fig. Qualitative changes in the intestinal microflora in calves with acute catarrhal bronchopneumonia of varying severity

significance requires additional studies. In this regard, we believe that the study of intestinal microflora in animals during inflammatory and infectious processes requires further scrupulous investigation.

CONCLUSION

The paper gives a detailed description of the intestinal tract microbiota in calves with acute catarrhal bronchopneumonia with varying degrees of severity of the infectious process. It has been shown that the qualitative and quantitative composition of the intestinal microbiome in calves with compensated acute catarrhal bronchopneumonia does not differ from clinically healthy animals. It has been established that significant quantitative and qualitative shifts occur in the intestinal biotope of animals in case of subcompensated acute catarrhal bronchopneumonia, indicating the dysbiosis. Thus, the fecal samples showed a reliable increase in the number of the following genera populations: *Enterobacter*, *Citrobacter*, *Klebsiella*, *Proteus*, *Pseudomonas*, *Clostridium* and *Candida*, against the background of a highly reliable decrease in the genera *Lactobacillus* and *Bifidobacterium* by 17.56 and 17.18%, respectively, when compared with the indicators of clinically healthy calves. Even more significant quantitative and qualitative dysbiotic shifts in the intestinal tract microbiota were observed in calves with the most severe decompensated form of acute catarrhal bronchopneumonia. Thus, the fecal samples from diseased animals showed a reliable increase in the number of the following genera populations: *Escherichia*, *Enterobacter*, *Citrobacter*, *Klebsiella*, *Proteus*, *Pseudomonas*, *Staphylococcus*, *Streptococcus*, *Bacillus*, *Clostridium* and yeast fungi (genus *Candida*) when compared with the indicators of animals in the control group. This was revealed against the background of a highly reliable decrease in fecal samples of experimental animals of the *Lactobacillus* and *Bifidobacterium* genera populations by 37.5 and 32.7%, respectively, as compared with the control group. It should be noted that in case of subcompensated and decompensated acute catarrhal bronchopneumonia, the representatives of the genera *Klebsiella*, *Pseudomonas* and *Clostridium* were found in samples of feces of calves, unlike healthy animals and animals with a mild degree of pathology.

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Situational analysis on porcine diseases: general risk assessment and prioritization of epizootic threats to biosecurity systems of pig establishments in the Russian Federation

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ABSTRACT

The results of the situational analysis on porcine diseases in the Russian Federation and the expert assessment prioritizing the list of porcine pathogens significant for the pig industry of the country are presented. The method applied to analyse the expert estimates in the situational analysis allows for rapid assessment and interpretation of the situation with identification of priority diseases to be further addressed. The calculations demonstrated the sufficient degree of agreement among the experts (coefficient of concordance $W = 0.61$), and Pearson's chi-squared test statistic $\chi^2 = 51.33$ (≥ 21.02607) indicated that the concordance is not random and the results can be used in subsequent studies. The specific features of epizootiology of the agents of African swine fever, classical swine fever, porcine reproductive and respiratory syndrome that can impact the effectiveness of biosecurity systems of pig establishments, as well as further ways for improving biosecurity management measures are discussed. The overall risk for the pig industry in the Russian Federation that is associated with external sources is currently characterized as permanently high, requiring maintaining risk management measures at the pig establishments by both the managerial staff of the establishments and the State Veterinary Service. It is recommended that biosecurity measures against external threats should focus on diseases such as African swine fever (weight $\lambda = 0.52$), porcine reproductive and respiratory syndrome ($\lambda = 0.071$), classical swine fever ($\lambda = 0.068$) and infections considered emerging for the Russian Federation ($\lambda = 0.05$) according to the weights based on the expert estimation results. The biosecurity systems of the establishments should equally address other threats significant for the pig industry of the country: swine enzootic pneumonia, porcine pleuropneumonia (*Actinobacillus pleuropneumoniae*), Aujeszky's disease, streptococcosis (*Streptococcus suis*), porcine circovirus infection, foot-and-mouth disease, leptospirosis, transmissible gastroenteritis, cysticercosis ($\lambda = 0.02 \ldots 0.05$). The improvement of the governmental policy for eradication of African swine fever, porcine reproductive and respiratory syndrome, classical swine fever (including the substantial modification of the existing official pig turnover control, zoning, diagnosis and prevention quality, as well as the implementation of biosecurity standards) is the most significant factor, without which the disease eradication perspective is questionable.

Keywords: porcine diseases, epizootic situation, pig industry, biosecurity, veterinary and sanitary measures

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Ситуационный анализ по болезням свиней: общая оценка рисков и приоритизация эпизоотических угроз для систем биозащиты свиноводческих предприятий в Российской Федерации

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РЕЗЮМЕ

Представлены результаты ситуационного анализа по болезням свиней в Российской Федерации и экспертной оценки, в которой приоритизирован список значимых для промышленного свиноводства страны патогенов. Использованный способ оценки экспертного мнения в ситуационном анализе позволяет быстро реализовать и интерпретировать ситуацию, выделяя приоритеты по болезням для дальнейшего обсуждения. Произведенные расчеты показали достаточный уровень согласованности мнений экспертов (коэффициент конкордации $W = 0{,}61$), а расчетный критерий согласия Пирсона $\chi^2 = 51{,}33$ ($\geq 21{,}02607$) указывал на то, что конкордация не случайная и результаты могут использоваться в дальнейших исследованиях. Обсуждены особенности эпизоотологии возбудителей африканской чумы свиней, классической чумы свиней, репродуктивно-респираторного синдрома свиней, способные повлиять на эффективность систем биозащиты свиноводческих предприятий, а также дальнейшие пути по улучшению мер управления биозащитой. Совокупный риск для промышленного свиноводства в Российской Федерации со стороны внешних источников в настоящей ситуации охарактеризован как перманентно высокий, требующий поддержания мер управления рисками на свиноводческих предприятиях как администрацией, так и государственной ветеринарной службой. Меры биозащиты для противодействия внешним угрозам рекомендовано акцентировать на таких заболеваниях, как африканская чума свиней (вес $\lambda = 0{,}52$), репродуктивно-респираторный синдром свиней ($\lambda = 0{,}071$), классическая чума свиней ($\lambda = 0{,}068$) и эмерджентных для Российской Федерации инфекциях ($\lambda = 0{,}05$) соответственно полученному весу по итогам экспертной оценки. Остальным значимым для свиноводства страны угрозам: энзоотическая пневмония свиней, актинобациллезная плевропневмония свиней, болезнь Ауески, стрептококкоз (*Streptococcus suis*), цирковирусная инфекция свиней, ящур, лептоспироз, трансмиссивный гастроэнтерит свиней, цистицеркоз ($\lambda = 0{,}02 \ldots 0{,}05$) – представляется возможным уделить равное внимание в системах биозащиты предприятий. Наличие государственной политики эрадикации африканской чумы свиней, репродуктивно-респираторного синдрома свиней, классической чумы свиней (с основательным изменением существующего официального контроля оборота поголовья, зонирования, качества диагностики и профилактики, внедрения стандартов биозащиты) является наиболее значимым фактором, без которого перспектива искоренения болезней сомнительна.

Ключевые слова: болезни свиней, эпизоотическая ситуация, свиноводство, биозащита, ветеринарно-санитарные меры

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INTRODUCTION

Biosecurity, as defined by the World Organisation for Animal Health (WOAH), is a set of measures and managerial solutions designed to reduce the risk of introduction, establishment and spread of diseases, infections or infestations to, within and from an animal population. The set of measures is also aimed at reducing microbial/viral load, blocking pathogen transmission routes inside the establishment and preventing infection introduction from the outside. It includes specific and non-specific measures such as maintenance of the animal disease free status of the establishment (sanitation of facilities, feeds, water, personnel and animal hygiene, veterinary treatments); compliance with appropriate practices of animal farming, sanitary treatment of premises, vehicles, fomites, feeding lines and water pipelines (cleaning, washing and disinfection); prevention of infection introduction (ensuring that the staff members of the establishment, as well as animals, feeds, equipment, etc. introduced to the establishment comply with the relevant veterinary and sanitary requirements, the implementation of disinfection, insect and rodent control measures). As part of animal disease control activities, the biosecurity of establishments globally serves to gain the time required for early detection of an infectious agent in the production flow and at the start of the implementation of disease eradication measures in the zone or country in order to prevent an epizootic.

The most important thing in this regard is the availability of information on external threats (animal disease situation in the territory of location, seasonal disease absence/presence in the populations of domestic and wild animals and birds), as well as on factors predisposing to changes in the extent of external threats (orientation of the livestock industry of the region, feed production and animal product processing; orientation of the quarantine policy of the veterinary authority of the region; seasonal, socially and economically determined activity of various groups of urban and rural population, the structure of protected livestock and their contact patterns). Studying the specific features of the epizootiology of porcine diseases in the pig industry and prioritizing their significance will allow for the development of adequate biosecurity measures for the pig production sector [1].

Therefore, the description and assessment of external epizootic threats to pig establishments and prioritization thereof remains an urgent topic for situational analysis within the framework of discussing the arrangement and development of biosecurity measures for pig establishments in the Russian Federation.

MATERIALS AND METHODS

The assessment of external epizootic threats to the pig industry was carried out in the form of situational analysis with discussion of the characteristics of pathogens, which made it possible to identify gaps in biosecurity measures. Official data on porcine diseases, data from open official sources and specialized mass media were used for the work [2, 3].

Estimates were obtained through a survey of experts of the Laboratory and Diagnosis Centre (3 persons) and Information Analysis Centre (4 persons who are the authors of this paper, except N. Ye. Baskakova) of the Federal Centre for Animal Health. The authors of the paper (5 persons) summarized and discussed the results, described special aspects relevant for infection management based on the prioritized list.

The experts ($m = 7$) assessed the degree of significance of threats for the domestic pig industry by assigning them a rank. In total, 13 threats ($n = 13$) were assessed: 12 diseases/pathogens and 1 category – emerging diseases (exotic for the Russian Federation and not reported previously). The threat considered to be the most significant by an expert was ranked 13. When several factors were recognized as equally significant, they were assigned an equal rank. On the basis of the data from the questionnaire-based survey, a summary matrix of ranks was compiled, the ranks given by the experts were rearranged without changing the opinion of the experts. The reformatted rank matrix was used to make a ranked list of sums of the ranks and mean ranks for 13 threats; based on the sums of the ranks, the weights of the threats were calculated, and the survey matrix was converted into a matrix of transformed ranks. The evaluation of the results of the expert survey was carried out by calculating Kendall's coefficient of concordance (W) [4] for cases where there are tied ranks (identical rank values among the estimates from one expert), using the following formula:

$$W = \frac{S}{\frac{1}{12} m^2 (n^3 - n) - m \sum T_i},$$

where S is the sum of squared deviations of all ranks of each object of expert assessment from the mean value;

n is the number of assessed threats;

m is the number of experts;

T_i is the number of ties (types of repeated elements) among the estimates from the i-th expert:

$$T_i = \frac{1}{12} (\Sigma (t_l^3 - t_l)),$$

where t_l is the number of elements in the l-th tie for the i-th expert (the number of repeated elements).

The significance of Kendall's coefficient of concordance was evaluated using Pearson's chi-squared test statistic (χ^2). The weights of the assessed threats (λ) were obtained by converting the survey matrix into a matrix of transformed ranks (using the formula $S_{ij} = X_{max} - X_{ij}$, where $X_{max} = 13$).

The prioritization results were presented graphically in the form of a ranked list of threats and a diagram based on the weights (λ) reflecting threat significance, as well as through the discussion of the most significant threats.

RESULTS AND DISCUSSION

The following are epizootically significant threats, including transboundary ones, to the pig industry: 1) anthrax; 2) foot-and-mouth disease (FMD); 3) Aujeszky's disease (AD); 4) classical swine fever (CSF); 5) African swine fever (ASF); 6) porcine reproductive and respiratory syndrome (PRRS); 7) swine vesicular disease (SVD); 8) Nipah virus infection; 9) cysticercosis; 10) swine brucellosis; 11) rabies; 12) trichinellosis; 13) tuberculosis; 14) transmissible gastroenteritis (TGE); 15) porcine epidemic diarrhea (PED); 16) swine erysipelas; 17) swine enzootic pneumonia (*Mycoplasma hyopneumoniae*); 18) porcine parvovirus (PPV) infection; 19) porcine circovirus (PCV) infection; 20) porcine pleuropneumonia (*Actinobacillus pleuropneumoniae*, APP); 21) Teschen disease; 22) Glässer disease (*Haemophilus parasuis*); 23) streptococcosis (*Streptococcus suis*); 24) leptospirosis; 25) Seneca Valley virus infection; 26) swine dysentery; 27) swine influenza; 28) opportunistic bacterial infections [2, 3, 5, 6].

According to official data on porcine diseases for 2022, cases of swine erysipelas (1), rabies (2), chlamydiosis (2), mycoplasmosis (3), pasteurellosis (4), pseudomoniasis (6), trichinellosis (6), echinococcosis (6), Aujeszky's disease (10), tuberculosis (16), porcine parvovirus infection (20), leptospirosis (42), edema disease (ED) (383), colibacillosis (2,394) and African swine fever (6,626) were reported in the pig and wild boar populations in the Russian Federation [2].

Anthrax, rabies, trichinellosis, tuberculosis and brucellosis in pigs are subject to control by the veterinary authorities of the Russian Federation in accordance with the surveillance pattern that has historically proven to be reliable and that covers all epizootiologically significant areas. Thus, the country has comprehensive measures in place to ensure protection of both animals and humans. The situation for these porcine diseases in the Russian Federation as a whole is stable (controlled risk with sustained surveillance and prevention), the epidemic thresholds have not been exceeded in the past 3 years (from 2020 to the 1st quarter of 2023) [2]. It is expectable that at a particular establishment, the risk level would be most heavily influenced by the veterinary surveillance coverage of domestic and wild pig populations in the pig farming areas, isolation of populations and vaccination quality, where applicable. In case of detection of any of these diseases in the industrial pork production chain, immediate measures shall be introduced to identify and eliminate the source. Regardless of the local significance of these diseases for the establishments, in view of risks to humans, the significance of these diseases is assumed to be high and is not discussed further.

The results of the assessment of 13 main threats ranked by the experts according to their significance for the pig industry of the country (Fig.) showed that ASF has the prevailing priority based on the relative weight (λ) (52%).

Besides, PRRS (7.1%) and CSF (6.8%) occupy a relatively significant place. Infections considered emerging for the Russian Federation (5%) also have a higher priority than other threats (swine enzootic pneumonia, APP, AD, streptococcosis (*S. suis*), PCV infection, FMD, leptospirosis, TGE, cysticercosis), which were assessed as being of moderate or low priority to the pig industry of the country

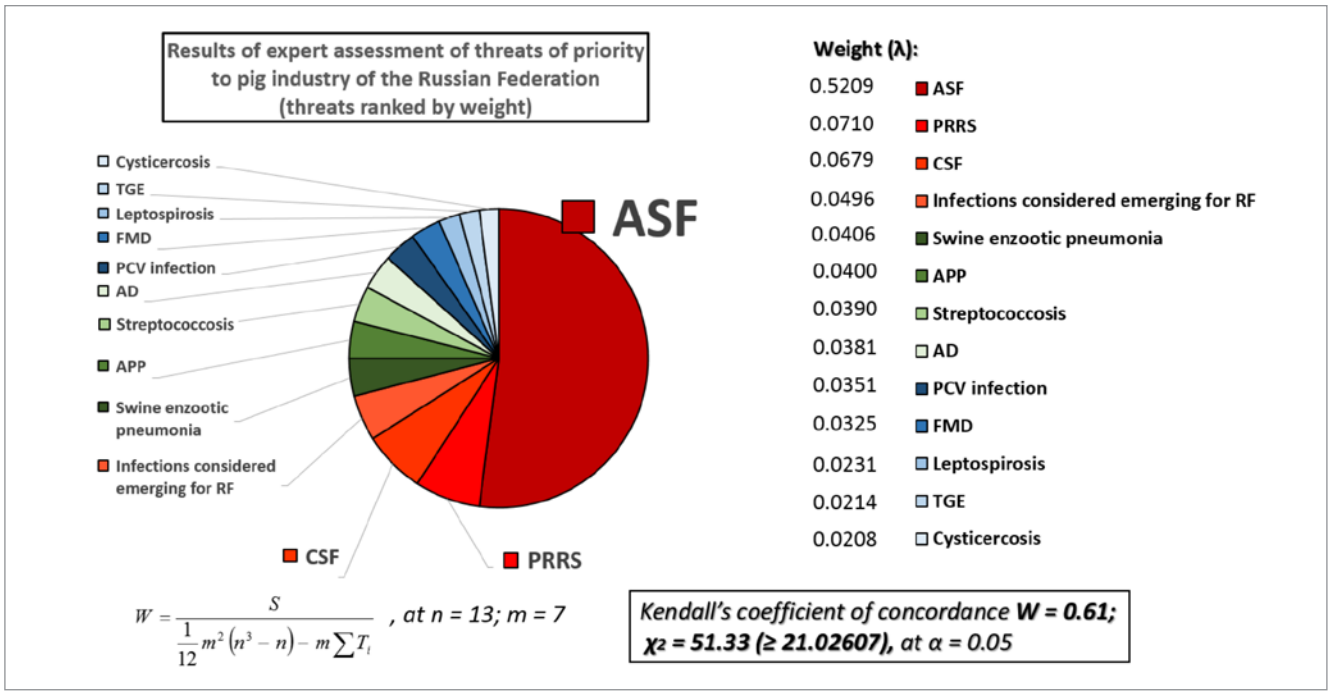

Fig. Results of assessment of threats (n = 13) ranked by experts (m = 7) according to their significance for the pig industry of the Russian Federation

(relative weight < 5%). Kendall's coefficient of concordance $W = 0.61$ demonstrates the sufficient degree of agreement among the experts, and the calculated chi-squared statistic $\chi^2 = 51.33$ (≥ 21.02607) indicates that the concordance is not random and the results can be used in subsequent studies. The Figure shows that there are three pathogens prioritized as significant external threats, so we will focus on the discussion of ASF, PRRS and CSF (Table).

African swine fever. By 2023, an ASF panzootic had overtaken 36 countries of the world, including wide areas in Eurasia. Russia has been facing the problem of ASF virus spread in domestic and wild pigs for 16 years [2, 3]. There is no commercially available ASF vaccine that meets the WOAH recommendations regarding safety and effectiveness requirements. The major factor contributing to the transboundary spread of the infection is the human factor (in our country, it is also officially recognized as the main factor according to Resolution of the Federation Council of the Federal Assembly of the Russian Federation No. 207-SF of 28 June 2017[1]), which in some cases can include not only neglect of and intentional disregard for biosecurity rules, lack of competencies, but also a protracted delay in carrying out preventive and eradication activities. Special attention is paid to studying possible ASF virus vectors, including mechanical ones, which, in the light of the global trends of changes in climate and environmental factors, is a task that is gaining relevance for the Russian Federation [7]. The detection of African swine fever virus in susceptible animals in ASF free areas, as well as the detection of ASF virus genome in the ready-to-eat product samples may indicate a continuing trend of ASF persistence.

The factors that contributed to ASF spread in the Russian Federation by 2013 included the diverse structure of pig farming in all federal districts of the Russian Federation and a high proportion of backyards [15], which declined significantly as of 2023. During the ASF epizootic of 2007–2023, the number of compartments and the sizes of herds in the indoor-keeping pig production systems in the Russian Federation were increased, biosecurity plans were introduced. However, population management and biosecurity management were not established systematically along the entire chain. Therefore, it was not possible to completely reduce the increased risk of the disease spread correlating with an increase in the number of farms/links and the expansion of pig and pork turnover in the context of the formation of the "grey market" of animals and meat outside and around the production systems.

The pork production system existing in the Eastern European countries is a combination of large and small pig farms, which is typical for ASF endemic countries [16]. ASF eradication in Spain, Portugal and the Czech Republic went hand in hand with a complete reshaping of the government policy of the disease surveillance, pig population recording, implementation and control of zoning, qualitative changes in diagnosis and establishment biosecurity. The assessment of risk in the biosecurity systems of the establishments plays a key role in the consideration of ASF risk [17], and compliance with the WOAH recommendations on ASF compartmentalization principles can serve as a mechanism for the establishments to contain the spread of the disease [18]. The main components of ASF control are timely and accurate diagnosis, stamping out of infected herds, establishment of restriction zones and tracing of possible contacts. In the future, ASF control should focus on enhanced restrictive measures, compensation of losses, tracing, wild boar control programmes, strict hygiene and biosecurity measures [19, 20, 21]. Activities undertaken to ensure the biosecurity of systems/establishments are aimed at isolating pigs kept there from wild boars [22, 23] and domestic pigs kept in the backyards.

Taking into account the opinions of foreign and domestic experts and summarizing the experience of pork

[1] https://base.garant.ru/71706886/?ysclid=lz81cejes4538929535 (in Russ.)

producers in implementing effective measures to prevent ASF in the pig industry [24, 25, 26], the following recommendations can be made.

1. The origin and movement of feeds, animals, vehicles and other objects, rodents, birds and persons entering the territory of a pig production establishment and having contact with pigs and fomites should be controlled by the biosecurity system of the establishment.

2. Monitoring of the area around the farms is required to demonstrate ASF absence and to maintain the ASF free status of the boundaries/zones. The involvement of backyards in pork production and pig turnover against the background of rising meat prices will remain a factor influencing the unauthorized pig and pork turnover.

3. The use of non-invasive samples from animals and laboratory control of the effectiveness of cleaning, washing and disinfection procedures may be recommended for early detection of ASF virus in the production flow.

4. Future vaccines against ASF shall not only be safe for the pig population and protect animals from death, but shall also be highly effective for routine use in the pig industry (they shall not reduce weight gain or cause abortions and shall prevent the circulation of field and vaccine strains in the pig population). The recognition of the safety and effectiveness of ASF vaccines shall take into account the international standards laid down by the WOAH.

Porcine reproductive and respiratory syndrome. Considerable attention is paid to the prevention of and protection against PRRS [27, 28, 29], given the high contagiousness of the pathogen, its ability to spread between pig farms over considerable distances through airborne transmission, with pigs, feeds and contaminated fomites and to

Table
Situation for priority epizootic threats to pig industry in the Russian Federation

Threat	Situation in the Russian Federation: year of the last reported cases / vaccination / RF status	Special aspects relevant for infection management
ASF	2023 (104 outbreaks: 66 outbreaks in the domestic population and 38 outbreaks in the wild population) / vaccination: none / federal measures to control ASF (Decree of the Government of the Russian Federation No. 1159 of 29 September 2018[2]); compartmentalization (Order of the Ministry of Agriculture of the Russian Federation No. 482 of 11 May 2023[3]); regionalization (Order of the Ministry of Agriculture of the Russian Federation No. 635 of 14 December 2015[4])	Very high priority ($\lambda = 0.52$) 1. The human factor predominates in the epizootiology of genotype II ASF in the Russian Federation. 2. It is necessary to control the implementation of measures aimed at the prevention of contacts between livestock kept at the establishment and environmental objects, including possible mechanical vectors. 3. Surveillance in the populations around and within the establishment shall take into account the possibility of latent infection. Period covered by the retrospective study during the investigation – up to 60 days. Within-herd R_0: from 7.46 (5.68–9.21) to 9.8 (3.9–15.6); between-herd R_0: from 1.65 (1.42–1.88) to 2.5 (2.0–3.0) [8, 9]. Where small farms prevail: within-herd R_0: 10 (1.1–30.0); between-herd R_0: from 1.41 to 10.8. 4. The absence of an effective and safe vaccine; the leading role of diagnostic activities in the production flow and during zoning.
PRRS	2021 (4 outbreaks in the domestic population) / 12,836,888 animals vaccinated / there is no federal programme in place; the current instruction on PRRS control regulates the use of PRRS vaccines for preventive purposes (Order of the Ministry of Agriculture of the Russian Federation No. 625 of 26 October 2020[5]); regionalization; compartmentalization	High priority ($\lambda = 0.071$) 1. Airborne transmission and transmission through fomites between establishments may be underestimated. 2. Vaccination is an effective tool, but only as part of the set of preventive and diagnostic measures. 3. R_0 within seropositive herds: from 3.3 (2.9–4.3) to 7.1 (3.5–10.6) [10, 11]. R_0 for vaccinated against type 1 PRRS ($R_0 < 1$): from 0.3 (0.05–0.96) [12] to 0.53 (0.19–0.76) [13]; for type 2 PRRS – unknown [11]. 4. The need for monitoring (genotyping) of circulating viruses in the vaccinated herds.
CSF	2018 (1 outbreak in the wild population) / 91,679,227 animals vaccinated / the Russian Federation has no official CFS free status recognized by the WOAH; regionalization; compartmentalization	High priority ($\lambda = 0.068$) 1. Effective indirect and direct transmission and spread of the infection. Within-herd R_0: from 3.39 (1.54–7.45) to 7.77 (4.68–12.9) [14]. 2. Vaccination of domestic pigs in the Russian Federation has been enhanced in the last 5 years. The vaccine reliably protects against outbreaks and the development of clinical signs in pigs. There were no outbreaks in domestic pigs and wild boars in 2018–2023. 3. The need for monitoring of circulating CSF virus isolates and vaccination effectiveness in vaccinated pig herds and wild boar populations of the Russian Federation regions. CSF virus circulation in the vaccinated animals is extremely undesirable and dangerous. 4. Monitoring of vaccination quality at the establishment and in the population around the establishment is important for CSF management.

R_0 (basic reproduction ratio) is a metric of infection reproduction (indicator of contagiousness). For all diseases, the control of animal introduction to the establishment, feeds, fomites, disinfection, disinsection and deratization are significant for security management. There are no consolidated biosecurity requirements/rules/standards for pig establishments.

[2] https://base.garant.ru/72065765/?ysclid=lz81dxcy83438154883 (in Russ.)
[3] https://base.garant.ru/406957068/?ysclid=lz81g64bk5614070677 (in Russ.)
[4] https://www.garant.ru/products/ipo/prime/doc/71260810/?ysclid=lz81h1rvb3947478382 (in Russ.)
[5] https://base.garant.ru/74832093/?ysclid=lz81jjcega297725170 (in Russ.)

affect virtually all pig population groups. In cold climates (at t < 0 °C), PRRS virus is able to survive outside the host for a long time, which contributes to contamination and mechanical transmission of the virus with fomites. Livestock transportation vehicles, personnel vehicles, footwear and other objects may come into contact with the PRRS agent in potentially contaminated places (infected farms, commercial truck wash facilities, slaughterhouses, changing and showering facilities, animal care product warehouses, semen transportation, transshipment facilities for tools, food products for the personnel, etc.). In cold weather, PRRS virus can be spread with contaminated objects over long distances (at least up to 50 km) [11, 30], which is relevant for most of the Russian Federation territory with prolonged cold seasons.

According to official data, PRRS spread in the Russian Federation is limited to individual cases detected in different areas every year, which indicates an underestimation of more widespread latent circulation of the virus in the domestic pig population and the insufficiency of current measures to completely eradicate the disease. PRRS poses a great danger to large fully integrated pig establishments [29]. This is confirmed by the results of a study of a large-scale PRRS outbreak that occurred in 2020 on 24 out of 30 farms of a large pig production company located in four Oblasts: the Voronezh, Lipetsk, Tambov and Penza Oblasts. The disease was caused by two variants of wild subtype 1 type 1 PRRS virus (PRRSV-1-1) predominant in Europe and the Russian Federation. The clinical signs varied depending on the pig production stage rather than on the virus variant. Non-compliance with biosecurity measures, including the movement of animals from infected farms, contributed to the spread of the disease. Before spreading across the production system (pig farms, sow farms and fattening farms), PRRSV-1-1 variants were introduced to the region around 2019 [31]. It was at the same time that the co-circulation of type 1 PRRSV-1 (European type) and type 2 PRRSV-2 (North American type) was first reported in Russia. According to the observations of S. Raev et al. and data from the veterinary laboratories, PRRSV-2 is circulating in both the European and Asian parts of the country, and the detection of new subtype 2 of type 1 PRRSV (PRRSV-1-2) during the study of the 2019 outbreak in Siberia confirmed the wide territorial expansion of PRRSV-1-2 in Russia [32].

Only few comparative data exist concerning the pathogenicity of the two types of PRRS virus and mixed infection under the field conditions in the Russian Federation; however, it is known that the isolates of PRRSV-1-1 (including the Russian group of viruses), PRRSV-1-2 and PRRSV-1-3 differ significantly in pathogenicity. Today, PRRSV-1 is a group of genetically diverse isolates from Eastern Europe, Belarus and Russia [33, 34].

The quantitative assessment confirmed that the disease caused by genotype 1 virus can be controlled with vaccines, but they provide only partial protection [11]. Vaccination constrains the dynamics of PRRSV transmission in the population and, according to some sources [12, 13], PRRS basic reproduction ratio within vaccinated herds is $R_0 < 1$ [from 0.3 (0.05–0.96) to 0.53 (0.19–0.76)], which means that the epizootic will eventually die out. On the other hand, some findings show that despite an almost two-fold decrease in mean R_0 values in the groups of vaccinated piglets, R_0 confidence intervals for vaccinated (2.43–39.7) and non-vaccinated (5.93–32.3) animals vary with significant overlap [35], which requires further research. In non-vaccinated endemic/seropositive populations within farms, R_0 ranges from 3.3 (2.9–4.3) to 7.1 (3.5–10.6) [10, 11]. The differences in R_0 values are explained by many factors, including the genetic difference between the vaccine strain and the challenge strain, the environmental/farm conditions (quality of segregation/ventilation) under which pigs are kept, and the difference in strains, to which pigs are adapted on farms.

Despite the absence of the federal target/sectoral programme for PRRS, the existing tools such as the compartmentalization of pig farms adopted in the country, the regionalization of the Russian Federation territory for contagious diseases and the current Veterinary Rules concerning PRRS (Order of the Ministry of Agriculture of the Russian Federation No. 625 of 26 October 2020[5]) are in line with contemporary understanding of PRRS in pig industry and aimed at maintaining PRRS freedom, including through the application of vaccines, and surveillance using diagnostic methods that take into account the vaccination status and allow for strain differentiation (paragraphs 8, 18 and 38 of the Veterinary Rules concerning PRRS). According to the WOAH recommendations (Article 15.3.3 [36]), a country or a farm is considered to be free from PRRS only when no vaccination against PRRS is practised, irrespective of the diagnostic capabilities of PRRS surveillance currently in place in the country.

The epizootiology of PRRS is far from being fully understood, but the available knowledge is sufficient to identify, at least qualitatively, the main sources of the infection on the farm, as well as to detect the main mechanisms of the virus transmission within the farm. The share of each transmission route in the virus introduction in different epizootiological scenarios is still unknown. The eradication of PRRS virus in the pig production systems is a difficult practical task, not just for an individual farm, and in most cases this process covers considerable administrative territories and involves all economic entities/farms [27].

PRRS eradication is a developed set of managerial solutions, including, as a rule, vaccination at the first stages. A combination of strict compliance with biosecurity measures and well-designed vaccination programmes can be useful for PRRS control at both the establishment and regional levels [11], which can be recommended.

Classical swine fever. CSF remains one of the most significant transboundary viral diseases of pigs worldwide and is taken into consideration when setting up surveillance, vaccination and biosecurity systems for farms. The basic reproduction ratio (R_0) for CSF virus, although varying across pig groups and establishments, always remains high: for weaner pigs between-pen R_0 and within-pen R_0 are 7.77 (4.68–12.9) and 100 (54.4–186), respectively, and for adult (slaughter) pigs they are 3.39 (1.54–7.45) and 15.5 (6.20–38.7), respectively [14].

CSF eradication on individual farms located in the CSF infected region/the region where vaccination is practised is an extremely costly policy, which cannot eliminate CSF risk for the region. CSF eradication in the pig production systems of the countries has historically included, as a rule, the depopulation of pigs across the entire regions, the implementation of zoning with progressive CSF eradication

[5] https://base.garant.ru/74832093/?ysclid=lz81jjcega297725170 (in Russ.)

in the zones and only then gaining the official CSF free status recognized by the WOAH. After the introduction of strict control measures, several countries have managed to eradicate CSF; nevertheless, CSF is present, at least sporadically, in most regions of the world with considerable pig production. Due to the proven effectiveness of vaccines currently used in the world against the circulating strains of CSF virus, immunization remains the main measure for pig death prevention [37].

The importance of compliance with the basic protective measures against CSF (control of animal and feed introduction, control of vaccination and immune status of animals, tracing of pigs, etc.), regardless of the reliability and quality of the vaccines used, is confirmed by the results of analysis of data from the surveillance implemented recently in various countries. The surveillance results for the period from 2014 to 2020 showed that in Ecuador, the risk factors that most strongly influenced the odds of CSF occurrence were swill feeding (odds ratio OR 8.53), time until detection (OR 2.44), introduction of new pigs (OR 2.01) and lack of vaccination (OR 1.82). The spatiotemporal model showed that vaccination reduces the risk of CSF spread by 33%. The complexity of CSF control programmes and the importance of improving the surveillance system were highlighted [38]. In 2019, in Laos, the system for porcine disease (brucellosis, PRRS and CSF) surveillance at the slaughterhouses using serological methods did not allow for the differentiation between seropositive vaccinated and infected pigs, which confirmed the need for animal tracing as the basis for surveillance programme implementation in the absence of DIVA strategy [39]. The problems related to the imperfection of diagnostic tests and sampling applied for CSF surveillance in the Russian Federation are also reported, and in this regard the usefulness of implementing both DIVA strategy and appropriate evaluation of vaccination programmes is underlined [37, 40]. It is most advisable that the biosecurity systems of the establishments should be based on the effective surveillance of CSF virus and animal immune status in the production flow.

No CSF cases have been reported in domestic pigs in the Russian Federation since 2015. The country practises routine vaccine prevention in domestic pigs [2]. According to data from the Cerberus Information System as of 1 January 2024, the Vladimir Oblast is declared to be CSF free with vaccination and the Chukotka Autonomous Okrug is declared to be CSF free without vaccination. All other regions of the Russian Federation have no official CSF free status (the status is undefined). Today, the vaccine reliably protects pigs against clinical CSF, and considerable vaccination coverage over the past 5 years contributes to the absence of outbreaks on the farms. Despite vaccination, the risk of latent circulation of the virus in the population remains high.

The main recommendations for CSF risk management at the establishments where vaccination is practised are as follows: 1) the origin and movement of feeds, animals, vehicles and other objects, rodents, birds and persons entering the territory of a pig production system and having contact with pigs and fomites should be controlled by the biosecurity system of the establishment; 2) the control of vaccination effectiveness (tests for immunity level) and targeted monitoring for early detection of possible CSF virus circulation in the immunized animals at the establishments should be an integral part of the surveillance system.

CONCLUSION

The method applied to analyse the expert estimates in the situational analysis allows for rapid assessment and interpretation of the situation with identification of priority diseases to be further addressed.

The overall risk for the pig industry in the Russian Federation that is associated with external sources is currently characterized as permanently high. At present, African swine fever is the absolute priority in terms of threat significance for the pig industry. Kendall's coefficient of concordance $W = 0.61$ demonstrates the sufficient degree of agreement among the experts, and the calculated chi-squared statistic $\chi^2 = 51.33$ (≥ 21.02607) indicates that the concordance is not random and the results can be used in subsequent studies. It is recommended that biosecurity measures against external threats should focus on diseases such as African swine fever ($\lambda = 0.52$), porcine reproductive and respiratory syndrome ($\lambda = 0.07$), classical swine fever ($\lambda = 0.068$) and infections considered emerging for the Russian Federation ($\lambda = 0.05$), which require the adoption and maintenance of risk management measures by both the pig establishments and the State Veterinary Service. The biosecurity systems of the establishments should equally address other assessed external threats significant for the pig industry of the Russian Federation: swine enzootic pneumonia, porcine pleuropneumonia (*Actinobacillus pleuropneumoniae*), Aujeszky's disease, streptococcosis (*S. suis*), porcine circovirus infection, foot-and-mouth disease, leptospirosis, transmissible gastroenteritis, cysticercosis ($\lambda = 0.02…0.05$). The specificities of the epizootiology of the pathogens may underlie gaps in the existing measures; therefore, the measures should be subject to regular re-assessment and adjustment within the biosecurity systems.

The improvement of the governmental policy for the eradication of African swine fever, porcine reproductive and respiratory syndrome, classical swine fever (including the substantial modification of the existing official pig turnover control, zoning, diagnosis and prevention quality, as well as the implementation of biosecurity standards) is the most significant factor, without which the disease eradication perspective is questionable, including given the continuing internal risk of pathogen persistence in the pig population of the Russian Federation or of a particular farm, which is constrained by vaccination that protects against the development of clinical signs. Any weakening / failure of vaccination regimens is likely to result in outbreaks and spread of infection.

At present, there are no consolidated requirements/rules for the biosecurity of the pig establishments. There is also no consolidated standard that establishes biosecurity requirements for production processes at the pig establishments. To a great extent, certain provisions of the current rules concerning diseases partly address the biosecurity of the establishments, which is aimed at preventing animal diseases, and are complemented by the existing requirements for compartmentalization, current rules for keeping pigs at the establishments and veterinary and sanitary requirements for livestock facilities. Excessiveness in the existing rules of requirements and standards related to the biosecurity of the establishments, in the light of their target orientation, cannot be confirmed, nor can their effectiveness and sufficiency for the biosecurity systems of the establishments be attested, especially in the absence

of mandatory requirements for the biosecurity systems of the pig establishments.

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Biochemical blood parameters in platinum fox females and males in ontogenesis

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ABSTRACT

Currently, veterinarians pay much attention to the diagnostic examination of animals, including animals kept on fur farms. Blood is the main material used for such examinations. Changes in its composition allows veterinary practitioners to identify disorders in various systems and organs of animals, as well as to assess metabolism in animals. Results of biochemical tests of serum samples from platinum fox males and females of different age groups and comparative assessment thereof are presented. The levels of aspartate aminotransferase (U/L), alanine aminotransferase (U/L), alkaline phosphatase (U/L), total protein (g/L), albumin (g/L), urea (mmol/L), creatinine (μmol/L), *α*-amylase (U/L), cholesterol (μmol/L) were determined. Aspartate aminotransferase levels in females at the age of 6 months were lower by 69% than that ones in males. Increase in aspartate aminotransferase by the age of 6 months helps animals to accumulate body weight before winter. Sexual differences in the alkaline phosphatase levels were detected in 1.5-month-old kits: alkaline phosphatase levels were higher by 21.05% in males than in females. By the age of 6 months, the alkaline phosphatase levels decreased in both males and females. The decline in alkaline phosphatase level with the age is associated with participation of this enzyme in the development of animal skeleton during ontogenesis. From the age of 4 months, the growth and development of the skeleton slows down, and by the age of 6 months the animals gain the size and body weight of adult animals. Urea and creatinine levels in foxes of both sexes increased during their growth, but remained within the reference limits. Changes in urea levels in blood can be caused by feeding excessively high-protein or excessively low-protein diets. The total protein content in sera from 4 month-old males and females decreased by 32.51 and 43.24%, respectively, compared with that one in sera from animals at the age of 1.5 months, and increased at the age of 6 months up to the level observed at the age of 4 months. According to the literature, rather rapid stabilization of protein metabolism is a biological feature of many mammals born in spring, their growth rate is accelerated and, in general, the maturity phase is shortened.

Keywords: platinum foxes, alkaline phosphatase, cholesterol, amylase, urea, creatinine, albumins, aspartate aminotransferase, alanine aminotransferase

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Биохимические показатели крови у самок и самцов лисиц платинового окраса в онтогенезе

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РЕЗЮМЕ

В настоящее время ветеринарные специалисты уделяют много внимания диагностическому обследованию животных, в том числе в пушном звероводстве. Основным материалом для таких обследований является кровь. Изменение ее состава позволяет врачам-практикам выявить нарушения в работе различных систем и органов животных, а также оценить метаболизм. В представленных материалах отражены результаты биохимических исследований сыворотки крови самок и самцов лисиц платинового цветового типа разных возрастных групп, проведена их сравнительная характеристика. Определяли

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уровень аспартатаминотрансферазы (Е/л), аланинаминотрансферазы (Е/л), щелочной фосфатазы (Е/л), общего белка (г/л), альбумина (г/л), мочевины (ммоль/л), креатинина (мкмоль/л), *α*-амилазы (Е/л), холестерина (мкмоль/л). Показатели аспартатаминотрансферазы у самок в возрасте 6 мес. были ниже, чем у самцов, на 69%. Увеличение аспартатаминотрансферазы к 6-месячному возрасту способствует накоплению массы тела в период подготовки зверей к зиме. У 1,5-месячных щенков выявлены половые различия в уровне активности щелочной фосфатазы: у самцов данный показатель выше, чем у самок, на 21,05%. К 6-месячному возрасту уровень щелочной фосфатазы понижался как у самцов, так и у самок. Снижение активности щелочной фосфатазы с возрастом животных обусловлено участием фермента в формировании скелета в процессе онтогенетического развития. С 4-месячного возраста рост и развитие скелета замедляется, а к 6 мес. звери приобретают размеры и массу тела взрослых животных. Показатели мочевины и креатинина у лисиц обоих полов в процессе роста животных увеличивались, но оставались в пределах референтных границ. Изменение количества мочевины в крови может наблюдаться при потреблении корма со слишком малым или чрезмерно большим количеством белка. Содержание общего белка в сыворотке крови у самцов и самок в возрасте 4 мес. снизилось на 32,51 и 43,24% соответственно по сравнению со значениями в 1,5 мес., а в возрасте 6 мес. показатели снова поднялись до уровня 4 мес. По литературным данным, относительно быстрая стабилизация белкового обмена является биологической особенностью, характерной для многих млекопитающих, рожденных весной, у них ускорен темп роста и в общем сокращена фаза достижения зрелости.

Ключевые слова: лисицы платинового окраса, щелочная фосфатаза, холестерин, амилаза, мочевина, креатинин, альбумины, аспартатаминотрансфераза, аланинаминотрансфераза

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INTRODUCTION

The analysis of biochemical blood parameters allows us to identify changes in animal and human organisms, as well as to assess the specificity of changes characteristic of a certain pathology.

Despite numerous and long-term studies, blood composition remains understudied, especially its changes depending on the sex and age of fur animals. This could be accounted for different blood sample collection procedures and different methods used for blood testing [1].

Analysis of scientific and methodological literature shows that the main publications were made 40–60 years ago, and during this, albeit seemingly short, time, there have been quite noticeable changes: adaptation of fur animals to cage rearing conditions has continued, the fur animal dietary pattern has deteriorated resulting in blood composition changes. Therefore, even the reference values should be reviewed every 10–15 years. The transaminase levels change first [2]. It should be noted that different methods use different units of blood parameter measurement that hampers comparison of results from tests carried out in different years, especially with a difference of 40–60 years. Fur animal blood tests are described mainly in V. A. Berestov's monographs published more than half a century ago [3, 4]. These monographs are rare and it is difficult to find them in libraries. Therefore, V. A. Berestov's monograph on fur animal clinical biochemistry was republished 20 years ago, but it contained old data without their clarifications and changes [5].

Biochemical reactions in the body are closely interrelated, metabolic reactions are highly coordinated. Taking into account various biological functions of blood, study of blood biochemical parameters in ontogenesis is of current importance. So, the study was aimed at testing of sera from platinum foxes for biochemical parameters in ontogenesis.

MATERIAL AND METHODS

Platinum foxes kept on OOO "Vyatka" fur animal breeding farm located in the Kirov Oblast were used for testing. Blood samples were collected from 10 fox females and 10 fox males at the age of 1.5; 4 and 6 months. The foxes were fed a diet generally used on this fur farm.

The tests were carried out at the Veterinary Laboratory of the Professor Zhitkov Federal State Budgetary Russian Research Institute of Game Management and Fur Farming. Blood samples for biochemical tests were collected from the lateral subcutaneous vein of the lower part of a fox leg into a special tube containing a clot activator before morning feeding of the foxes. Then, sera prepared by centrifugation at 2,000 rpm for 15 minutes. The levels of aspartate aminotransferase (AST, U/L), alanine aminotransferase (ALT, U/L), alkaline phosphatase (U/L), total protein (g/L), albumin (g/L), urea (mmol/L), creatinine (μmol/L), *α*-amylase (U/L), cholesterol (μmol/L) were determined using the semi-automatic BiochimSA analyzer (USA) and commercial test-kits.

The results were processed using the licensed MS Excel (Office 2019) IBM SPSS Statistics 26 software.

Mann – Whitney U-test, non-parametric statistical test, was used for each group to assess the homogeneity of the groups and the reliability of the differences in means between the groups. Differences between the compared groups were considered statistically significant at $p < 0.05$. The data were summarized in terms of mean (*M*), standard error of the mean (*m*) [6].

The works were performed in compliance with the international principles laid down in the Declaration of Helsinki for Animals (Declaration of Helsinki, 2013), Directive 2010/63/EC of the European Parliament and of the Council of the European Union on the protection of animals used for scientific purposes as well as in accordance with the Rules for experimental animal handling (Annex to Order of the Ministry of Health of the USSR No. 755 of 12 August 1977) and methodological guidelines for conducting scientific and field experiments on feeding of fur animals [7, 8, 9].

RESULTS AND DISCUSSION

Biochemical tests of sera for AST and *α*-amylase showed that there was no difference in AST and *α*-amylase levels between fox females and fox males at the ages of 1.5, 4 and 6 months. AST levels in fox females at the age of 6 months increased by 5.25% as compared to that ones in fox females at the age of 1.5 months ($p \leq 0.05$). In fox males, AST levels decreased by 13.68% by the age of 6 months ($p \leq 0.05$).

ALT levels in fox females determined at the age of 1.5 months were 37.28% higher than in fox females at the age of 4 months ($p \leq 0.05$). In fox males at the age of 1.5 months ALT level was 37.57% higher than in fox males at the age of 4 months ($p \leq 0.05$). AST/ALT ratio, known as De Ritis ratio, varies from 0.9 to 1.73 U/L in healthy animals [10]. The AST/ALT ratio in foxes of different sexes at the age of 1.5 months was the same, and in fox females at the age of 4 months was 31.58% higher than in fox females at the age of 1.5 months, but at the age of 6 months the AST/ALT ratio became similar to that one at the age of 1.5 months (Table 1).

The AST/ALT ratio increased by 53.13% ($p < 0.05$) in fox males at the age of 6 months, as compared to that one in fox males at the age of 4 months. Berezina Yu. A. et al. studied ALT and AST activities in silver-black foxes in postnatal ontogenesis. The ALT level increased more than AST level in foxes throughout the studied period of the fox life. It should be noted that the enzyme activity in fox females was lower than in fox males. ALT and AST levels in blood increased during fox ontogenesis, and the highest AST and ALT levels were detected in adult foxes. Thus, ALT level increased by 27% ($p < 0.01$) and by 34% ($p < 0.05$) in adult fox females and adult fox males, respectively, as compared to that ones in 2 months-old kits as well as AST level increased by 16 and 26% ($p < 0.01$), respectively, according to literature data [11]. The AST level became 12.04% lower in platinum fox males at the age of 6 months than in the animals at the age of 1.5 months that was not reliable. The maximum transaminase content observed in fur animals in autumn (6 months of age), during the preparation for the cold season, contributed to an active increase in body weight in animals that also correlated to the results of other studies [12, 13]. ALT (cytoplasmic enzyme) activity increases in case of mild hepatocyte injury, and AST (mitochondrial enzyme) activity increases in case of significant hepatocyte injury [14, 15].

Tests for alkaline phosphatase showed that the alkaline phosphatase level was 33.65% higher in fox females at the age of 1.5 months than in animals at the age of 4 months and 2 times higher than in foxes at the age of 6 months ($p \leq 0.05$). The alkaline phosphatase activity decreases with age of animals, since the enzyme is involved in bone calcification reducing with the age. Fur animals grow rapidly, so they acquire the size and weight of adult animals by the age of 6 months [10, 16]. The age

Table 1
Biochemical blood parameters in platinum fox females and fox males during postnatal ontogenesis

Parameters	Age of fox females and fox males					
	1.5 months		4 months		6 months	
	♀	♂	♀	♂	♀	♂
AST, U/L	$^{A}39.18 \pm 2.00$	$38.80 \pm 3.05^{**}$	37.73 ± 1.06	34.50 ± 0.74	$^{A}41.24 \pm 1.97^{*}$	34.13 ± 1.35
ALT, U/L	$68.16 \pm 2.53^{*}$	$^{B}73.64 \pm 11.17^{**}$	49.65 ± 2.23	$^{B}53.53 \pm 2.90$	69.98 ± 8.47	70.78 ± 7.91
De Ritis ratio (AST/ALT), U/L	0.57	0.52	0.75	0.64	0.58	0.98
Alkaline phosphatase, U/L	$^{A}112.78 \pm 7.55^{*}$	$^{C}136.52 \pm 8.42^{***}$	$^{A}74.83 \pm 7.45$	$^{C}100.70 \pm 6.53^{***}$	$^{A}55.94 \pm 2.93$	$^{C}64.65 \pm 2.89$
Urea, mmol/L	4.52 ± 0.16	4.04 ± 0.64	3.93 ± 0.40	3.85 ± 0.53	5.38 ± 0.59	$5.80 \pm 0.42^{**}$
Creatinine, μmol/L	38.94 ± 4.17	47.74 ± 5.44	$68.40 \pm 1.47^{**}$	$77.65 \pm 3.57^{*}$	$77.14 \pm 1.26^{*}$	62.85 ± 7.68
Total protein, g/L	$88.71 \pm 3.36^{*}$	$83.72 \pm 2.88^{**}$	50.35 ± 2.26	56.50 ± 2.82	83.24 ± 3.14	84.55 ± 13.13
Albumin, g/L	43.00 ± 1.59	$42.34 \pm 0.64^{**}$	39.05 ± 1.33	39.05 ± 0.81	38.32 ± 1.43	42.98 ± 2.10
Cholesterol, μmol/L	7.21 ± 0.47	5.84 ± 0.18	6.67 ± 0.77	6.06 ± 0.09	6.00 ± 0.41	5.98 ± 0.63
α-amylase, U/L	534.38 ± 43.59	569.42 ± 48.59	563.25 ± 24.12	651.83 ± 36.52	619.38 ± 18.79	600.80 ± 35.47

* $p \leq 0.05$ – between ♀ at the age of 1.5 and 4 months; ** $p \leq 0.05$ – between ♂ at the age of 1.5 and 4 months;
[A] *$p \leq 0.05$ – between ♀ at the age of 1.5 and 6 months; [B] ** $p \leq 0.05$ – between ♂ at the age of 1.5 and 6 months;
[C] *** $p \leq 0.05$ – between ♀ and ♂ at the age of 4 months.

of the animals is directly proportional to the alkaline phosphatase level. Sex differences in the alkaline phosphatase level were found in 1.5-month-old kits. It was higher by 21.05% ($p \leq 0.05$) in fox males than in fox females. By the age of 6 months, the alkaline phosphatase level became similar in fox females and fox males.

Urea level increased by 19.03 and 43.56% ($p \leq 0.05$) in fox females and fox males, respectively, during their growth but remained within the reference limits (Table 2). Urea is synthesized in the liver, travels with blood to the kidneys, then, it is filtered through the vascular glomerulus and excreted in the urine. Urea plays an osmotically active role in the body. Urea accumulation facilitates edema development in parenchymal organs (lungs, liver, kidneys, pancreas, spleen, thyroid gland), central nervous system, myocardium, subcutaneous tissue. Tests for urea are important since it contributes to about half of the total residual nitrogen as liver converts ammonia into urea, being the final product for protein metabolism. It should also be noted that nitrogen deficiency significantly increases urea reabsorption in the kidneys. Changes in urea levels (decrease or increase) in animal blood can be caused by feeding excessively high-protein or excessively low-protein diets. It is known that the urea level in dogs fed with dry food is about 1.7 times lower than in dogs fed with canned meat [17, 18].

Tests for creatinine showed that its level increased by 75.65% ($p \leq 0.05$) and by 62.65% ($p \leq 0.05$) in fox females and in fox males at the age of 4 months, respectively, as well as by 98.1% ($p \leq 0.05$) and by 31.65% ($p \leq 0.05$) in fox females and in fox males at the age of 6 months, respectively, as compared to creatinine levels in 1.5-month-old animals. Serum creatinine is the most widely used functional biomarker of kidney function. Its concentration is quite stable and mainly depends on the total muscle mass. Creatine is synthesized in the liver from guanidine acetic acid, released into the blood stream and travels to the muscle cells where it is phosphorylated and transformed into phosphocreatine to be taken up to produce the energy required for muscle contractions. Dehydrated creatinine, being a non-threshold substance, is excreted in the urine. The creatinine level in the blood mainly correlates with the muscle mass and the kidney excretory ability. In case of chronic renal failure, an increase in creatinine level in the blood is accompanied by an increase in the concentration of other residual nitrogen components and, first of all, urea. A similar pattern is observed in case of urinary tract blockage [19, 20].

Protein including its fractions (albumin and globulins of several types) at a certain quantitative and structural ratio is one of the major components of the blood. Proteins play an important role in maintaining the colloidal osmotic pressure of the blood plasma. The circulating blood volume remains constant and the formed elements remains suspended owing to the protein ability to drag and retain water. In platinum fur animals the total protein content in sera from 4-month-old fox males and fox females were by 32.51 and 43.24 % ($p \leq 0.05$) lower, respectively, as compared with that one in sera from animals at the age of 1.5 months, and increased at the age of 6 months up to the level observed at the age of 4 months. According to some researchers [21], protein metabolism in mammals born in the spring season is relatively faster stabilized, that is a biological feature of such animals. Such animals grow faster and reach their maturity within a shortened period.

Tests for albumin levels showed no significant difference in both fox females and fox males of different age groups. Albumin is a homogeneous plasma protein containing a small amount of carbohydrates. Albumen is the most important fraction and amounts for more than 40–60% of the total serum protein. In domestic animals, albumin accounts for 35 to 50% of the total serum protein. Albumin is the main protein maintaining intravascular colloidal osmotic pressure, that prevents plasma from leaving capillaries [14, 18, 19]. In our case, albumin concentration in both fox females and fox males practically remained unchanged (Table 1) showing no statistical difference, the albumin levels were within the reference

Table 2
Reference limits for biochemical blood parameters in platinum fox females and fox males in ontogenesis

Parameters	Age of fox females and males					
	1.5 months		4 months		6 months	
	♀ min/max	♂ min/max	♀ min/max	♂ min/max	♀ min/max	♂ min/max
AST, U/L	31.50/47.00	28.80/45.30	35.00/39.70	33.50/36.70	36.00/45.30	30.90/36.40
ALT, U/L	60.20/78.80	36.10/106.20	44.10/53.60	47.40/61.30	42.80/84.20	57.60/90.10
Alkaline phosphatase, U/L	80.90/135.40	109.80/160.00	59.60/94.70	8.70/112.50	49.60/65.50	60.10/72.70
Urea, mmol/L	4.30/5.30	3.30/5.30	2.80/4.60	3.00/5.20	3.90/7.40	4.70/6.50
Creatinine, μmol/L	29.90/54.00	34.80/63.10	64.00/70.10	70.50/86.30	73.70/81.30	46.60/76.80
Total protein, g/L	72.30/93.90	76.20/93.50	47.10/56.70	51.90/63.80	75.30/92.30	54.80/118.30
Albumin, g/L	38.10/48.00	40.60/44.00	36.60/42.20	37.70/41.10	33.80/41.40	36.90/46.00
Cholesterol, μmol/L	5.49/8.95	5.44/6.46	5.31/8.39	5.85/6.22	5.00/7.10	5.10/7.80
α-amylase, U/L	417.00/717.40	459.30/703.00	521.30/631.80	554.90/731.70	571.70/672.50	522.20/680.80

limits (Table 2). Changes in albumin concentration are observed during fasting, chronic gastroenteritis, when protein digestion and absorption are impaired, as well as chronic liver diseases (hepatitis, hepatodystrophy, cirrhosis) [17].

Tests for cholesterol showed that its level decreased by 7.49 and 16.78% in fox females at the age of 4 and 6 months, respectively, as compared to that one in fox females at the age of 1.5 months. Cholesterol is the most important structural component of cell membranes. It participates in cell permeability regulation and protects red blood cells from hemolytic toxins. Cholesterol is used for synthesis of steroid hormones, vitamin D_3, and bile acids. Cholesterol is synthesized in all cells of the body but cholesterol released in the blood stream is synthesized in hepatocytes and small intestine cells. The liver plays a key role in cholesterol synthesis and cholesterol catabolism. Changes in cholesterol levels are characteristic of such diseases and pathologies as hepatic disease (hepatitis, bile duct obstruction), nephrotic syndrome, hypothyroidism, chronic pancreatitis, obesity, vitamin deficiency [20, 22, 23].

CONCLUSIONS

Thus, tests of fox female and fox male blood for biochemical parameters in ontogenesis showed the following:

1. Alkaline phosphatase levels (U/L) were higher by 21.05% in fox males than in fox females starting from the age of 1.5 months. By the age of 6 months, the alkaline phosphatase levels decreased in both fox males and fox females. The decline in alkaline phosphatase level with the age is accounted for its participation in the animal skeleton development during ontogenesis. From the age of 4 months the skeleton growth and development slow down and by the age of 6 months the animals gain the size and body weight of adult animals.

2. Urea and creatinine levels in foxes of both sexes increased during the growth of animals, but remained within the reference limits.

3. The total protein content in sera from 4-month-old fox males and fox females decreased by 32.51 and 43.24%, respectively, as compared to that one in sera from animals at the age of 1.5 months. According to V. A. Afanasyev and N. Sh. Pereldik [21], rather rapid protein metabolism stabilization is a biological feature of many mammals born in spring; such animals grow faster and reach their maturity within a shortened period.

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First detection of recombinant variant of African swine fever virus in the Russian Federation (brief communication)

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ABSTRACT

As part of extensive molecular and genetic research into African swine fever virus isolates circulating in Russia, a recombinant variant with a mosaic genome structure has been identified. The one that caused an outbreak on a pig farm in the Primorsky Krai, in 2023. The characterized strain ASFV/Primorsky_2023/DP-4560.Rec demonstrates hemadsorption, active propagation in porcine primary macrophage cell culture, 99.9917% identity with the first recombinant isolates from the People's Republic of China, recovered in 2021. Recombination sites included 79 open reading frames homologous to genotype II isolates; 49 ones homologous to genotype I and 12 mixed ones. Testing biomaterial from dead pigs in real-time polymerase chain reaction showed no changes in sensitivity or specificity, despite significant genetic distinctions between the recombinant and genotype II isolates that are enzootic to the Russian Federation. However, in 2023, D. Zhao et al. reported on high virulence of the virus related variants as revealed by the challenge tests in domestic pigs. Given the accelerating rates of AFSV molecular evolution in the East Asian countries (China, Vietnam and the Far Eastern regions of Russia), it is required to improve control measures, general and specific prevention, national and international surveillance over the economically significant animal disease.

Keywords: African swine fever, recombinant variant, genotype I, genotype II, the Primorsky Krai, the Far East

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Первое выявление рекомбинантного варианта вируса африканской чумы свиней в Российской Федерации (краткое сообщение)

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РЕЗЮМЕ

В рамках проведения комплексных молекулярно-генетических исследований изолятов вируса африканской чумы свиней, циркулирующих в России, идентифицирован рекомбинантный вариант с мозаичной структурой генома, вызвавший вспышку заболевания на территории свинокомплекса Приморского края в 2023 г. Охарактеризованный штамм ASFV/Primorsky_2023/DP-4560.Rec обладал феноменом «гемадсорбции», высокой репродукционной активностью в первичных культурах клеток макрофагов свиней, 99,9917%-й идентичностью с первыми рекомбинантными изолятами из Китайской Народной Республики, выявленными в 2021 г. Сайты рекомбинации включали 79 открытых рамок считывания, гомологичных изолятам генотипа II, 49 – генотипу I и 12 смешанных. Исследование методом полимеразной цепной реакции в режиме реального времени биологического материала, отобранного от павших свиней, не показало изменения чувствительности и специфичности, несмотря на значительные генетические различия рекомбинанта в сравнении с изолятами генотипа II, энзоотичными для Российской Федерации. Однако D. Zhao et al. в 2023 г. сообщалось о высоковирулентных свойствах родственных вариантов вируса в острых опытах на домашних свиньях. В связи с нарастающими темпами молекулярной эволюции вируса африканской

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чумы свиней в странах Восточной Азии (Китае, Вьетнаме и дальневосточных регионах России) необходимо усовершенствование мер контроля, общей и специфической профилактики, внутреннего и интернационального надзора за экономически значимой болезнью животных.

Ключевые слова: африканская чума свиней, рекомбинантный вариант, I генотип, II генотип, Приморский край, Дальний Восток

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In 2023, African swine fever situation (ASF) in Russia was unfavourable. The ASF outbreaks were detected in populations of wild boars and domestic pigs both in backyards and on commercial pig farms in a number of subjects of the Russian Federation.

As part of the research project implemented by the Federal Centre for Animal Health, ASF virus isolate (ASFV/Primorsky_2023/DP-4560.Rec), recovered from an outbreak reported on a commercial pig farm in the Primorsky Krai (in May 2023; Settlement Pervomayskoye, Raion Mikhailovsky), was subjected to a comprehensive sequencing and phylogenetic analysis.

The obtained results were compared with the data from the open sources describing isolates recovered in 2018–2023 in the Primorsky Krai and other subjects of the Russian Federation. As reference samples we used sequences of ASFV genotypes I and II strains from the GenBank international database (i.e. OURT 88/3 – No. AM712240.1 strain and Georgia 2007/1 – No. FR682468.2 strain, respectively), as well as sequences of recombinant isolates (genotypes I and II) detected during ASF outbreaks in 2021–2022 in domestic pigs in the Provinces of Hainan, Inner Mongolia and Jiangsu (China) [1].

During the research, we identified ASF virus that is able to accumulate in porcine bone marrow cell culture at a titer of (8.2 ± 0.23) lg HAD_{50}/cm^3, demonstrating the phenomenon of hemadsorption.

Genome-wide sequencing of ASFV/Primorsky_2023/DP-4560.Rec and ASFV/Pig/Jiangsu/LG_China/2021 (recombinant variant registered in China in 2021) isolates demonstrated 99.9917% homology (Fig. 1).

Results of the genome-wide comparison of phylogenetic trees given in Figure 2 clearly demonstrate correspondence of the studied virus from the Primorsky Krai to a separate clade of recombinant isolates from China (2021–2022).

Formation of the recombination sites (79 open reading frames (ORF) belong to genotype II, 49 to genotype I, 12 are mixed) in the genome of ASFV/Primorsky_2023/DP-4560.Rec strain are shown in Figure 3.

The recombinant variant described by D. Zhao et al. in 2023 was characterized as highly virulent for domestic pigs [1]. It was also reported that pilot ASF vaccines manufactured from HLJ/18-7GD strain (developed by Harbin Veterinary Research Institute), protect from virulent genotype II virus but do not protect pigs from infection with the recombinant variant [2].

In 2023, the recombinant virus variant, similar to the previous ones, was detected in the northern provinces of Vietnam [3].

Currently, the Federal Centre for Animal Health researchers are studying biological properties

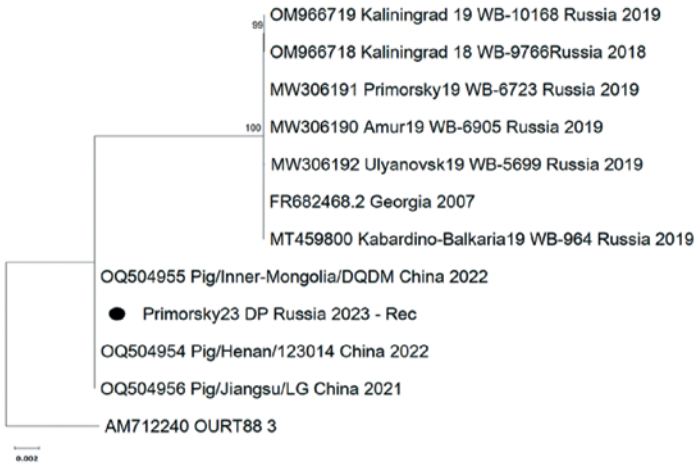

Fig. 2. Phylogenetic tree of ASFV/Primorsky_2023/DP-4560.Rec (●) and other ASF virus strains

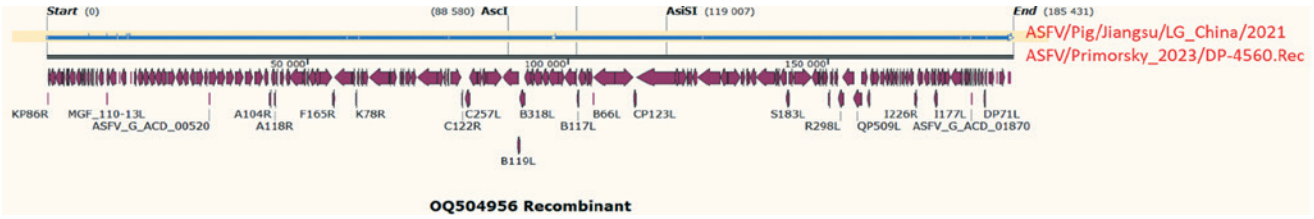

Fig. 1. Genome-wide map showing correspondence of sequences between ASF isolates ASFV/Primorsky_2023/DP-4560.Rec and ASFV/Pig/Jiangsu/LG_China/2021 (blue color shows homology between the sequences)

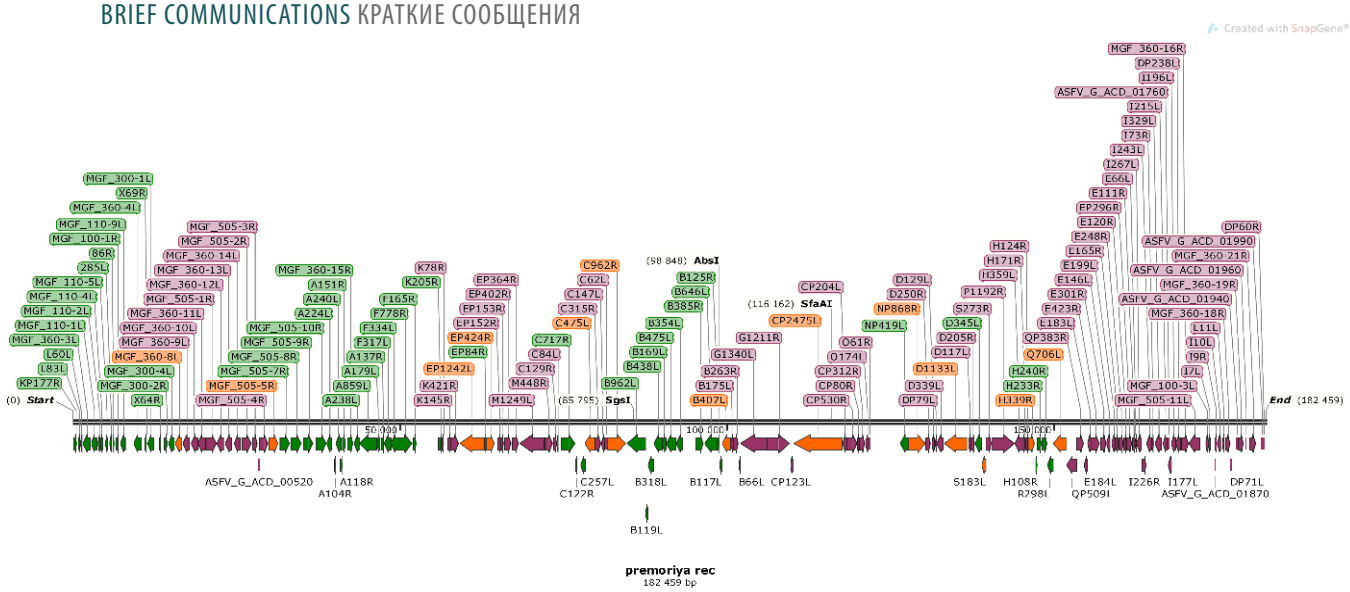

Fig. 3. Map of the annotated ORFs in ASFV/Primorsky_2023/DP-4560.Rec strain (ORFs with a high percentage of identity to genotype II sequence are given in purple; ORFs similar to genotype I are given in green; genes having sequence identical with genotype II, but mutations identical to genotype I are given in orange)

of ASFV/Primorsky_2023/DP-4560.Rec isolate, testing its properties in susceptible animals as well. Primary data suggest that the genome changes do not affect sensitivity and specificity of real-time polymerase chain reaction, which is widely used to diagnose the disease. The results of further and ongoing experiments will be reported additionally.

CONCLUSION

The data obtained re-confirm ASF transboundary nature, demonstrate vulnerability of the global pig industry and set up new requirements for vaccines being developed against this disease.

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ЦЕНТР ВОЗЖ ПО СОТРУДНИЧЕСТВУ В ОБЛАСТИ ДИАГНОСТИКИ И КОНТРОЛЯ ВИРУСНЫХ БОЛЕЗНЕЙ ЖИВОТНЫХ ДЛЯ СТРАН ВОСТОЧНОЙ ЕВРОПЫ, ЦЕНТРАЛЬНОЙ АЗИИ И ЗАКАВКАЗЬЯ

FAO REFERENCE CENTRE FOR FOOT-AND-MOUTH DISEASE
РЕФЕРЕНТНЫЙ ЦЕНТР ФАО ПО ЯЩУРУ

FAO REFERENCE CENTRE FOR ZOONOTIC CORONAVIRUSES
РЕФЕРЕНТНЫЙ ЦЕНТР ФАО ПО ЗООНОЗНЫМ КОРОНАВИРУСАМ