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Changes in the Number of White Blood Cells and Non-Specific Markers of Inflammation in the Body of Rabbits in Experimental Osteoarthritis of the Knee Joint

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Abstract. Osteoarthritis is the most common joint disease in animals, which is not always treatable. Both in veterinary and in human medicine, laboratory research methods are used to effectively diagnose this pathology. The purpose of this study was to establish the dynamics of markers of the acute phase of inflammation in the blood using experimental modelling of knee osteoarthritis in rabbits. Artificial reproduction of the pathological process in the knee joints of animals was performed by intra-articular administration of Yellow peel 2*5 ml. The BC-2800Vet automatic haematology analyser (China) was used to calculate the number of white blood cells and their subpopulations in the blood. The content of C-reactive protein in blood serum was found using enzyme-linked immunosorbent assay. The rate of erythrocyte sedimentation in the blood was investigated according to the Panchenkov method. Thus, on Day 7, a sharp increase in the number of white blood cells (2.7 times) and their subpopulations was noted in sick rabbits, which indicates an active inflammatory process of a systemic nature. On Day 14, their number in the blood of sick animals was characterized by a tendency to decrease, but it had not yet reached the values of the control group. On Days 21 and 28, all the indicators under study tended to decrease in sick animals. Specifically, the number of white blood cells and their subpopulations acquired reference values. The content of C-reactive protein significantly increased by 40.8 times in sick animals on Day 7 of the study and decreased by 1.7 times on Day 14 compared to its values at the previous stage. At the same time, on Day 28, this indicator increased by 19.3 times compared to the indicators of the control group of animals. The rate of erythrocyte sedimentation in the blood of sick rabbits significantly increased by 2.3 times compared to the control group. On Day 21, this indicator in the blood of sick rabbits decreased by 1.4 times compared to its values on Day 14 of the experiment. On the other hand, on Day 28, its value increased by 1.7 times compared to the control (4.3 mm/h). The results obtained will contribute to further targeted laboratory diagnostics of knee osteoarthritis in animals, which will ensure the proper effectiveness of therapeutic and preventive measures and improve their overall health

Keywords: laboratory diagnostics, leukogram, C-reactive protein, erythrocyte sedimentation rate

Introduction

Osteoarthritis is a dystrophic joint lesion that develops due to degeneration of the articular cartilage. Therewith, the inflammatory process occurs with damage not only to the cartilage, but also to all components of the joint.

This disease is a substantial problem in veterinary practice and the most common joint pathology in animals. The incidence rate is 2.5-20% in young animals and 35-55% in geriatric animals (according to statistics from the veterinary clinic "Dunai", Kyiv).

New effective methods and means of treatment that give rapid results and substantially reduce the risk of side effects are being actively developed [1-3]. Intra-articular administration of autologous enriched plasma is performed, stem cells, joint replacement, or arthroplasty are

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used. Therewith, the economic efficiency of using these methods is important.

In rabbits and other domestic animals, osteoarthritis is described by certain changes in morphological and biochemical parameters of the blood. These biomarkers can be used both in preclinical and clinical studies. The main purpose of such studies is to establish the diagnosis, figure out the stage of the disease and monitor its course, confirm the pharmacological mechanism of action, demonstrate the biological mechanism of action and predict the clinical outcome of the disease [4-6].

It is believed that most animals with osteoarthritis do not have specific changes in the results of blood and urine tests, except for cases of synovitis with significant effusion of synovial fluid into the articular capsule. Therewith, hypergammaglobulinemia is registered in the blood of sick animals, which provokes a substantial increase in the rate of erythrocyte sedimentation, leukocytosis develops with changes in the leukogram and an increase in the level of acute phase indicators – C-reactive protein, fibrinogen, etc. [4; 6; 7].

C-reactive protein is one of the most common test indicators used in clinical laboratory practice to assess, diagnose, and predict inflammation. However, its role in physiological processes is not yet fully understood. This protein is the most sensitive indicator of tissue damage due to inflammation, necrosis, and trauma. It is the very first of the acute phase proteins actively produced by hepatocytes under pathogenic factors. Its content is mainly regulated by circulating interleukin-6. C-reactive protein belongs to the family of pentraxins, shows a 1000-fold or greater increase in concentration during the development of pathological processes in tissues [7; 8].

The half-life of C-reactive protein from the blood is about 19 hours and stays stable regardless of the animal's health status. Its high sensitivity and ease of analysis have made it an ideal marker of inflammation. It was discovered in 1930 by William Tillett [8] and Thomas Francis [8] of Rockefeller University. The researchers reported that the third serological fraction, or "fraction C," isolated from patients infected with pneumococci, differed from the capsule polysaccharide and nucleoprotein fractions that were detected by a specific antibody response. Oswald Avery and McLean McCarthy [8], who postulated the "transformation principle" and the concept that genes are made up of DNA, also described that the levels of C-reactive protein as an "acute phase protein" were elevated in the serum of patients with inflammatory processes. Volanakis and Kaplan [8] later identified specific ligands capable of binding to C-reactive protein. It was found that this protein performs the function of conjugating pathogens and inducing their destruction by the complement system. It has also been investigated as a screening marker of inflammation, disease activity, and as a diagnostic supplement. The exact role of C-reactive protein as an acute phase protein requires further evaluation. Such information focuses on its diagnostic significance in various diseases, as well as on established and contradictory evidence for its physiological role [7; 8].

The half-life of serum C-reactive protein in both clinically healthy rabbits and animals treated with inflammatory stimuli is 4-6 hours [7; 8]. The content of C-reactive protein in the blood serum of rabbits during an experimental study of the pathological process immediately increases, and its peak level is noted on the 3rd day after inoculation. Thus, it is believed that serum C-reactive protein is an informative marker of inflammatory processes existing in the animal body [7; 8].

The purpose of this study lies in the investigation of changes in the number of white blood cells and their subpopulations, the content of C-reactive protein and the rate of erythrocyte sedimentation in the blood of rabbits in experimental osteoarthritis of the knee joint.

Literature Review

Osteoarthritis is a degenerative-dystrophic joint disease that occurs due to the gradual physical destruction of articular cartilage [9].

Osteoarthritis is also known as the most common type of degenerative joint disease (DJD) and develops more often with age. Changes in osteoarthritis usually occur slowly over many years, although there are exceptions. Inflammation and joint damage cause bone changes, destruction of tendons and ligaments, and destruction of cartilage, leading to pain, swelling, and deformity of the joint [9; 10].

Dogs of different breeds are prone to developing osteoarthritis due to poor breeding. Dog breeds such as Labrador, springer spaniel, German shepherd, golden retriever, Rottweiler, and Bernese Mountain Dog are more susceptible to this joint pathology [2; 3; 9].

Any dog can develop osteoarthritis, especially with age [6-8]. However, there are some factors that can lead to its development in animals of large breeds (German shepherd, Labrador, golden retriever). These include obesity, age, especially middle-aged and older dogs; long-term exposure to stress factors during sports activities (flyball, diving); injuries such as fractures or torn ligaments; hip or elbow dysplasia; infections affecting the joints (Lyme disease); unbalanced diet and genetics [8; 9].

Osteoarthritis is also a severe problem in horses, which can develop regardless of their breed and age. On the territory of Ukraine, there are certain problems with the diagnosis of this disease in horses at an early stage because not all veterinary clinics can conduct such in-depth examinations of animals.

Unfortunately, osteoarthritis is a rapidly progressive disease, and there are no effective medications. Stabilization of the patient's condition is focused on relieving pain, reducing the inflammatory process, improving the quality of life, and slowing down the development of the disease. Treatment of osteoarthritis is usually multimodal, i.e., diverse treatment methods are used simultaneously to achieve the best result [9; 11; 12].

To improve joint function, reduce inflammation, and slow the progression of damage, dietary supplements are prescribed to animals [11; 12]. Glucosamine and chondroitin are two common ingredients in joint supplements used in both dog and human treatments. These supplements reduce inflammation, promote healing, and increase water retention in the cartilage, which provides better cushioning for the joint. Green-lipped mussel (GLM) is another proven dietary supplement ingredient for treating joint pathology in both animals and humans, which contains beneficial nutrients such as omega-3 fatty acids, glycosaminoglycans, and antioxidants. It is a powerful anti-inflammatory agent that reduces pain and preserves joint function. Supplements are

often used is animal diet during the progression of osteoarthritis because they are safe for long-term use [12].

Therefore, the use of dietary supplements in animals and the control of pain sensitivity by introducing nonsteroidal anti-inflammatory drugs are the basis for the treatment of osteoarthritis. The most common medications for pain relief in the more severe form of osteoarthritis are nonsteroidal anti-inflammatory drugs, which can both reduce pain and the progression of inflammation in the joints. However, these preparations have significant side effects with prolonged use, especially in patients with poor liver or kidney function. That is why it is necessary to consider the risks and benefits of treating animals with nonsteroidal anti-inflammatory drugs for osteoarthritis. Therewith, regular morphological and biochemical blood tests should be performed [10; 11; 13].

Notably, keeping an optimal body weight of animals and an active lifestyle affects the activity of the joints. In overweight animals with osteoarthritis, an increase in pain is observed, and the processes of cartilage destruction are accelerated [10; 11].

Timely and effective laboratory diagnosis of this pathology in animals is relevant precisely because standard treatment protocols for osteoarthritis of animals do not give positive results, and even lead to complications from the digestive system, liver, and kidneys.

Materials and Methods

The study was conducted in the scientific laboratory of the I.O. Povazhenko Department of Surgery and Pathophysiology of the Faculty of Veterinary Medicine of the National University of Life and Environmental Sciences of Ukraine during 2021-2022.

The study material was 24 clinically healthy male rabbits with a body weight of 2.5 to 2.8 kg. They were divided into two groups: control and experimental with 12 animals each. Animals of the control group were intra-articularly injected with isotonic (0.9%) NaCl solution in a dose of 0.7 ml. Experimental development of osteoarthritis was modelled in the animals of the experimental group by intra-articular administration of a 4% solution of retinol acetate and kojic acid (Yellow Peel preparation, Medicare, Germany) in a dose of 0.7 ml. Intra-articular injections were performed using a 23 G needle (0.6*32 mm) at intervals of 7 days, twice.

During the simulation of experimental osteoarthritis, a clinical examination of animals was performed, as well as a study of the number of white blood cells and their subpopulations in the blood, the content of C-reactive protein and the rate of erythrocyte sedimentation.

Blood for these studies was taken from animals of the control and experimental groups on Days 7, 14, 21, and 28 after modelling experimental osteoarthritis of the knee joint.

Experiments using laboratory animals were conducted in compliance with the requirements of the "General ethical principles for conducting experiments on animals", approved by the National Congress on Bioethics (09/20/2004, Kyiv, Ukraine) [14] and the provisions of the "European Convention for the protection of vertebrates used for experimental and other scientific purposes" (Strasbourg, 1986) [15], the Law of Ukraine "On the Protection of Animals from Ill-treatment" [16; 17].

The groups of values of indicators in dynamics were compared according to the Student's parametric criterion with the determination of the arithmetic mean (M) and its error (m). Differences between the two indicators of the compared samples at $P < 0.05$, $P < 0.01$, and $P < 0.001$ were considered statistically significant [18].

Changes in the number of white blood cells and their subpopulations in the blood were evaluated using BC-2800VET automatic haematology analyser (Mindray Building, Keji 12th Road South, High-tech Industrial Park, Nanshan, Shenzhen 518057, P. R. China) [19], within three hours after sampling.

The content of C-reactive protein was examined in the blood serum of rabbits using the Vcheck kit (manufactured by Bionote, Korea). The Vcheck kit is based on a fluorescent enzyme-linked immunosorbent assay for quantitative measurement of C-reactive protein concentrations. The concentration of this protein was found using a BIONOTE Vcheck analyser (manufacturer Bionote, Korea) based on the calibration curve data [20].

The rate of erythrocyte sedimentation in the blood was investigated according to the Panchenkov method [21]. According to this method, an anticoagulant was used – a 3.8% solution of sodium citrate. A chemical solution was added to a test tube containing capillary blood and placed vertically for 60 minutes. Therewith, red blood cells settled to the bottom of the test tube. The time it takes for them to settle is considered the rate of erythrocyte sedimentation.

The list of 20 criteria corresponding to the diagnosis of rheumatoid arthritis classified by the American Rheumatism Association includes an indicator of increased erythrocyte sedimentation rate. Most rheumatologists believe that an important diagnostic criterion is a thorough examination, which should confirm inflammation of the synovial membrane of the joint (synovitis). However, the erythrocyte sedimentation rate is a useful indicator in case of a doubtful diagnosis [10; 11; 22].

Results and Discussion

In case of joint diseases, it is important in diagnosis not only to take an anamnesis and a clinical examination, but also laboratory tests – counting the number of leukocytes, changes in the leukogram, the level of the erythrocyte sedimentation rate and the content of C-reactive protein in the blood serum. The research results are consistent with the results of a study by other authors [11; 23; 24].

It was found that on Day 7 of the experiment, changes in the number of white blood cells and their subpopulations in the blood of rabbits of the experimental group were noted (Table 1).

Table 1. The number of leukocytes and their subpopulations in the blood of rabbits on Day 7 of the experiment with experimental osteoarthritis (M ± m, n = 12)

Indicator	Control group	Experimental Group	Reference values
White blood cells (g/l)	5.4 ± 0.1	14.6 ± 0.2***	5-13
Neutrophils (%)	48.2 ± 0.9	52.5 ± 0.6***	34-70

Table 1, Continued

Indicator	Control group	Experimental Group	Reference values
Eosinophils (%)	0.9 ± 0.2	8.8 ± 0.5***	0-2
Basophils (%)	0.0 ± 0.0	0.3 ± 0.2*	0-0.8
Lymphocytes (%)	50.2 ± 1.1	23.2 ± 1.1***	43-80
Monocytes (%)	0.8 ± 0.3	15.2 ± 0.5***	0-4

Note: *P < 0.05; ***P < 0.001 compared to the control group of rabbits.

Based on the results of the study given in Table 1, it follows that on Day 7 of the experiment, a significant 2.7-fold (p < 0.001) increase in the number of white blood cells in the blood of sick rabbits (experimental group) is observed compared to the animals of the control group. This indicates an acute inflammatory process in the body of rabbits of the experimental group. The number of neutrophils, eosinophils, basophils, and monocytes in their blood was also significantly higher, 1.1 times (P < 0.001), 9.7 times (P < 0.001), 33% (P < 0.05), and 19 times (P < 0.001), respectively, compared to the control animals. At the same time, the number of lymphocytes in the blood of sick rabbits was significantly less by 2.2 times (P < 0.001) compared to the control. These changes indicate an inflammatory process caused in response to preparation administration and activation of nonspecific immunity in experimental osteoarthritis in animals of the experimental group. In rabbits of the control group, these indicators corresponded to the limits of reference values.

Consequently, experimental osteoarthritis causes a general inflammatory process in the body of animals of the experimental group, which is also consistent with experimental data of S. Chandrashekara [7].

The results of the study of the number of leukocytes and their subpopulations in the blood of rabbits on Day 14 of the experiment with experimental osteoarthritis are presented in Table 2.

Table 2. The number of white blood cells and their subpopulations in the blood of rabbits with experimental osteoarthritis on Day 14 of the experiment (M ± M, n = 12)

Indicator	Control group	Experimental Group	Reference values
White blood cells (g/l)	6.0 ± 0.1	14.2 ± 0.2***	5-13
Neutrophils (%)	47.6 ± 1.0	49.8 ± 0.6	34-70
Eosinophils (%)	0.8 ± 0.2	2.5 ± 0.2***	0-2
Basophils (%)	0.0 ± 0.0	0.6 ± 0.2**	0-0.84
Lymphocytes (%)	50.9 ± 1.2	42.1 ± 1.5***	43-80
Monocytes (%)	0.7 ± 0.2	7.1 ± 0.3***	0-4

Note: **P < 0.01; ***P < 0.001 compared to the control group of rabbits

Table 2 suggests that on Day 14 of the experiment, the number of white blood cells in the blood of rabbits of the experimental group significantly increased by 2.4 times (P < 0.001) compared to the control group of animals. Therewith, the number of eosinophils, basophils, and monocytes significantly increased, respectively, by 3.1 times (P < 0.001), by 60% (P < 0.01) and by 10.1 times (P < 0.001) compared to the control group. However, the number of lymphocytes in the blood of rabbits of the experimental group significantly decreased by 1.2 times (P < 0.001). There was no statistically significant difference between the neutrophil count in the experimental and control groups of animals.

Such changes in white blood cells in the blood of rabbits of the experimental group indicate the activation of specific factors of the immune system on Day 14 of the experiment (in the study, this is a response to the introduction of the preparation, but its concentration decreases).

As data in Table 2 suggests, in the control group of animals, the indicators of the number of white blood cells and their populations in the blood correspond to the limits of reference values, which is consistent with the research results of E. Oohashi *et al.* [8]. The research results on the number of white blood cells and their subpopulations in the blood of rabbits on Day 21 of the experiment with experimental osteoarthritis are presented in Table 3.

Table 3. The number of white blood cells and their subpopulations in the blood of rabbits with experimental osteoarthritis on Day 21 of the experiment (M ± M, n = 12)

Indicator	Control group	Experimental Group	Reference values
White blood cells (g/l)	5.4 ± 0.1	12.6 ± 0.2***	5-13
Neutrophils (%)	43.3 ± 1.1	48.1 ± 0.9	34-70
Eosinophils (%)	0.5 ± 0.2	2.4 ± 0.3***	0-2
Basophils (%)	0.0 ± 0.0	1.5 ± 0.2***	0-0.8
Lymphocytes (%)	51.9 ± 1.0	45.9 ± 0.9***	43-80
Monocytes (%)	0.4 ± 0.2	1.5 ± 0.2***	0-4

Note: ***P < 0.001 compared to the control group of rabbits

According to the results presented in Table 3, on Day 21 of the study, the number of white blood cells in the blood of sick rabbits was significantly higher by 2.3 times (P < 0.001), eosinophils – by 4.8 times (P < 0.001), basophils – by 1.5% (P < 0.001), monocytes – by 3.8 times (P < 0.001) compared to the control. At the same time, the number of lymphocytes significantly decreased by 1.1 times (P < 0.001). The number of neutrophils tended to increase in the blood of rabbits of the experimental group by 1.1 times. Changes in the number of leukocytes and their subpopulations in the blood of sick rabbits indicate an inflammatory process caused by a foreign agent (in the conducted study, this is a response to the administration of the preparation at a reduced concentration).

In the blood of the control group of animals, indicators (Table 3) correspond to the limits of reference values, which indicates the absence of an inflammatory process in the animal body.

The results of the study of the number of white blood cells and their subpopulations in the blood of rabbits on Day 28 of the experiment with experimental osteoarthritis are presented in Table 4.

Table 4. The number of white blood cells and their subpopulations in the blood of rabbits with experimental osteoarthritis on Day 28 of the experiment (M ± M, n = 12)

Indicator	Control group	Experimental Group	Reference values
White blood cells, (g/l)	5.3 ± 0.1	10.8 ± 0.1***	5-13
Neutrophils (%)	42.9 ± 0.9	46.5 ± 0.9**	34-70
Eosinophils (%)	0.5 ± 0.2	1.3 ± 0.2*	0-2
Basophils (%)	0.0 ± 0.0	0.7 ± 0.2***	0-0.8
Lymphocytes (%)	56.2 ± 0.9	49.3 ± 0.9***	43-80
Monocytes (%)	0.4 ± 0.2	2.4 ± 0.3***	0-4

Note: *P < 0.05; ***P < 0.001 compared to the control group of rabbits

As data from the Table 4 suggests, on Day 28 of the experiment, the number of white blood cells in the blood of rabbits of the experimental group significantly increased by 2.0 times (P < 0.001), neutrophils – by 1.1 times (P < 0.01), eosinophils – by 2.6 times (P < 0.05), basophils – by 0.7% (P < 0.001) and monocytes – by 6.0 times (P < 0.001) compared to animals of the control group. At the same time, the number of lymphocytes in the blood of rabbits in the experimental group significantly decreased by 11 times (P < 0.001) compared to the control group. These changes indicate an inflammatory process in the animal body, but the more time passes from the date of the last injection of the irritant, the less pronounced this inflammatory process becomes. In the blood of rabbits of the control group, the indicators corresponded to the limits of reference values.

It is known from the literature that C-reactive protein is a sensitive marker and is used to monitor inflammatory processes [2]. In animals of the experimental group for osteoarthritis, the content of C-reactive protein depended on the stage of development of experimental osteoarthritis. As Figure 1 shows, the content of C-reactive protein in the blood serum of animals of the experimental and control groups differed significantly, which corresponds to the research results of A. Hillström [2].

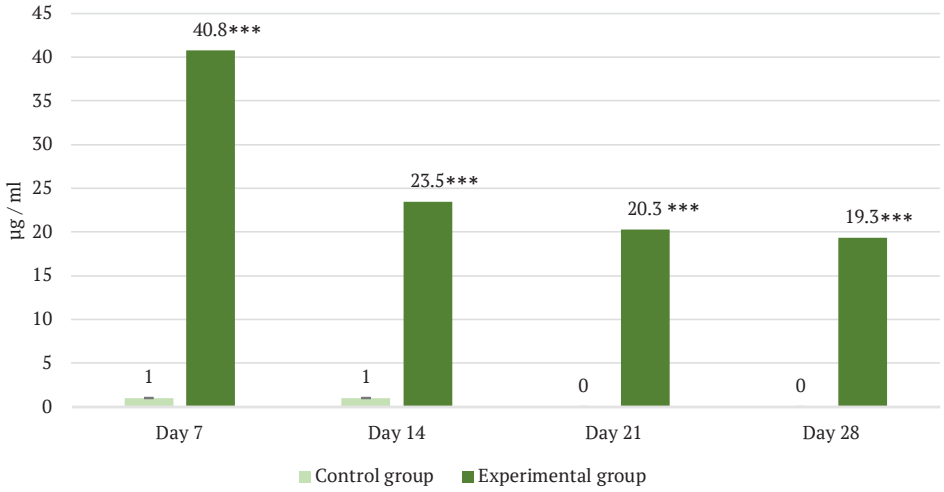

Figure 1. Evaluation of the level of C-reactive protein in the blood serum of rabbits of the control and experimental groups

Thus, on Day 7 of the study, the content of C-reactive protein in the blood serum of control rabbits was within the control values. In the blood serum of rabbits of the experimental group, the content of C-reactive protein significantly increased by 40.8 times ($P < 0.001$) compared to the control group (1 μg/ml), which indicates an acute inflammatory process in the animal body with experimental osteoarthritis.

On Day 14 of the study, the content of C-reactive protein in the blood serum of rabbits in the experimental group increased by 23.5 times ($P < 0.001$) compared to animals in the control group, where its concentration stayed unchanged and on average corresponded to the value of 1 μg/ml. Therewith, the content of this protein decreased by 1.7 times compared to that on Day 7 of the study. At the same time, in the control group of rabbits, the content of C-reactive protein in the blood serum was within the control values. This points to a decrease in the concentration of an irritating substance in the animal body, which was used to simulate experimental osteoarthritis.

On Day 21 of the study, the C-reactive protein content in the blood serum of rabbits in the control group corresponded to the range of reference values. In rabbits of the experimental group, the indicator of this protein content significantly increased by 20.3 times ($P < 0.001$) compared to animals of the control group, where it was 0 μg/ml. Notably, the concentration of this protein in the blood of rabbits of the experimental group was 1.2 times lower ($P < 0.001$) compared to the indicator on Day 14, as well as 2.0 times relative to Day 7.

On Day 28 of the experiment, the content of C-reactive protein in the blood serum of rabbits in the control group corresponded to the limits of reference values. In the blood serum of animals of the experimental group, this indicator increased by 19.2 times ($P < 0.001$) compared to the control (0 μg/ml). The content of C-reactive protein in the blood serum of rabbits of the experimental group decreased by 1.1 times compared to its value on Day 21 of the study. This indicates the course of an acute inflammatory process.

Thus, the study of the content of C-reactive protein in the blood serum of animals can be used for rapid diagnosis of inflammatory diseases of the joints. The presence of this inflammation marker in the blood serum of rabbits of the experimental group indicates that after modelling the inflammatory process in the knee joint during the first week of the study, an acute inflammatory process occurred as a reaction to an irritating substance. In the period up to the fourth week of research, the content of this protein decreased, but compared to its values in the control, it stayed elevated. The results of these studies are consistent with the data of other authors [2].

Therewith, a study was conducted to find the rate of erythrocyte sedimentation in the blood of rabbits with experimental osteoarthritis (Fig. 2). Thus, on Day 7 of the experiment, in the blood of animals of the experimental group, the value of this indicator increased by 2.3 times ($P < 0.001$) compared to that in animals of the control group (4.8 mm/h).

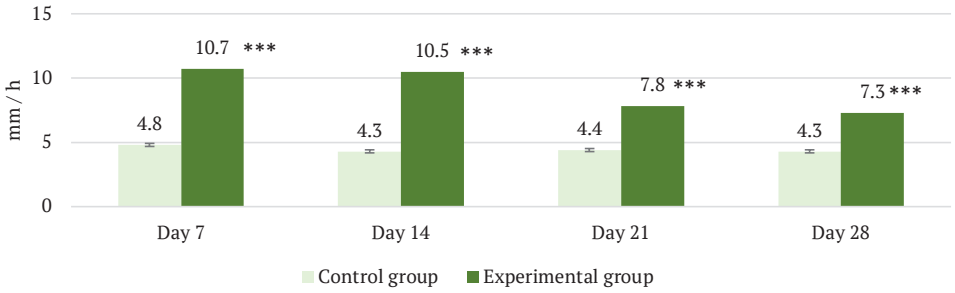

Figure 2. Changes in the rate of erythrocyte sedimentation in the blood of experimental rabbits

It is known from the literature sources that an increase in the rate of erythrocyte sedimentation indicates an inflammatory process in the animal body [21; 25; 26]. The mechanism of this phenomenon is explained by a decrease in the electrical charge of blood cells due to the adsorption of acute phase inflammatory proteins on their surface.

On Day 14 of the study, a 2.4-fold increase in the erythrocyte sedimentation rate ($P < 0.001$) was observed in the blood of animals of the experimental group compared to the control (4.3 mm/h). On Day 21 of the experiment, a 1.4-fold decrease in the value of this indicator was noted in the blood of these rabbits compared to the previous period. However, the rate of erythrocyte sedimentation in the blood of rabbits of the experimental group increased by 1.8 times ($P < 0.001$) compared to animals of the control group (4.4 mm/h).

On Day 28 of the study, a decrease in the rate of erythrocyte sedimentation in the blood of rabbits was noted compared to that on Day 21 of the study. Therewith, this indicator in the blood of rabbits of the experimental group increased by 1.7 times ($P < 0.001$) compared to animals of the control group (4.3 mm/h).

Thus, an increase in the rate of erythrocyte sedimentation in the blood of animals of the experimental group is connected with the fact that modelling experimental osteoarthritis in rabbits provokes not only a local, but also a systemic inflammatory process. The results of these studies are consistent with the data of other authors [21; 24].

Therefore, leukocytosis, an increase in the level of C-reactive protein, and an increase in the rate of erythrocyte sedimentation in the blood of rabbits with experimental osteoarthritis are a significant sign of the presence of an inflammatory process in the body, which is consistent with the results of the study by Legrand *et al.* [4].

Conclusions

It was found that in the blood of rabbits with artificial modelling of osteoarthritis of the knee joint, the development of leukocytosis is observed, which is especially pronounced on Days 7 and 14 of the experiment. An increase in the

number of leukocytes in the blood of sick animals indicates not only a local inflammatory process in this joint, but also a generalized nature of the pathology, to which the body's immune system reacts.

A significant increase in the number of neutrophilic granulocytes in the blood of sick animals during the experiment also indicates an inflammatory process in the body. The number of eosinophils in the blood of these rabbits increases by Day 7 and decreases by Day 28, which indicates a direct dependence of the synthesis of immune system cells in such animals in response to the irritating effect of intra-articular administration of retinol acetate and kojic acid. The increase in the number of basophils during the experiment is obviously caused by the active proliferation and differentiation of haematopoietic stem cells in the bone marrow of animals. The decrease in the number of lymphocytes in the peripheral blood of sick rabbits is probably explained by the accumulation of lymphocyte clones in the peripheral organs of the immune system (lymph nodes, spleen) to activate the synthesis of immunoglobulins and cytokines, which ensure the cooperation of cellular-humoral links of immunity in experimental osteoarthritis in the knee joint. Therewith, monocytosis is associated with the reaction of immune system cells to a local inflammatory process in the animal body.

The content of C-reactive protein in the blood serum of rabbits with experimental osteoarthritis of the knee joint increases by 40.8 times on Day 7 of the experiment and decreases on Days 14, 21, and 28.

In experimental osteoarthritis of the knee joint, the blood of rabbits shows an increased rate of erythrocyte sedimentation, which is also one of the markers of the inflammatory process in their body.

Further research in this area may accelerate the early diagnosis of osteoarthritis of the knee joint, which will improve the effectiveness of treatment in the preliminary stages of the development of the pathological process. This will also contribute to further quality control of therapy to improve the clinical condition of animals and timely prevent further joint destruction.

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Зміни кількості лейкоцитів і неспецифічних маркерів запалення в організмі кролів за експериментального остеоартрозу колінного суглобу

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Анотація. Остеоартроз є найпоширенішим захворюванням суглобів у тварин, яке не завжди піддається лікуванню. Як у ветеринарії, так і в медицині для ефективної діагностики цієї патології використовують лабораторні методи дослідження. Метою роботи було визначення динаміки маркерів гострої фази запалення в крові за експериментального моделювання остеоартрозу колінного суглоба в кролів. Штучне відтворення патологічного процесу в колінних суглобах тварин здійснювали шляхом внутрішньосуглобового введення препарату Yellow peel 2*5 ml. Для підрахунку кількості лейкоцитів та їх субпопуляцій в крові використовували автоматичний гематологічний аналізатор BC-2800Vet. Уміст С-реактивного білка у сироватці крові визначали за допомогою імуноферментного аналізу. Швидкість осідання еритроцитів у крові досліджували за методом Панченкова. Так, на 7 добу в хворих кролів відмічали різке підвищення кількості лейкоцитів (у 2,7 раза) та їх субпопуляцій, що вказує на активний запальний процес системного характеру. На 14 добу їх кількість в крові хворих тварин характеризувалася тенденцією до зменшення, проте вона ще не досягала значень показників контрольної групи. На 21 і 28 добу всі досліджувані показники в хворих тварин мали тенденцію до зменшення. Зокрема, кількість лейкоцитів та їх субпопуляцій набувала референтних значень. Уміст С-реактивного білка вірогідно підвищувався в 40,8 раза у хворих тварин на 7 добу дослідження та зменшувався в 1,7 раза на 14 добу порівняно з його значеннями на попередньому етапі. Водночас на 28 добу цей показник зростав у 19,3 раза порівняно з показниками контрольної групи тварин. Швидкість осідання еритроцитів у крові хворих кролів вірогідно зростала в 2,3 раза порівняно з контрольною групою. На 21 добу цей показник у крові хворих кролів знижувався в 1,4 раза порівняно з його значеннями на 14 добу експерименту. Натомість, на 28 добу спостереження його величина зростала в 1,7 раза порівняно з контролем (4,3 мм/год). Отримані результати сприятимуть надалі проведенню прицільної лабораторної діагностики остеоартрозу колінного суглобу в тварин, що забезпечить належну ефективність лікувально-профілактичних заходів та покращення загального стану їхнього здоров'я

Ключові слова: лабораторна діагностика, лейкограма, С-реактивний білок, швидкість осідання еритроцитів

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Influence of Different Environments on Oocyte Maturation and Development of Bovine Embryos *in Vitro*

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Abstract. According to the International Embryo Technology Society, the number of bovine embryos produced by *in vitro* fertilization technology is increasing every year. However, despite the large volumes of their production, the effectiveness of this method is still low and needs to be improved. Therefore, the purpose of this study was to compare the effectiveness of two commercial media – Origio Sequential Series (Origio, Denmark) and a panel of products manufactured by Minitube (Germany) in terms of oocyte maturation and development of bovine embryos *in vitro*. At the first stage of the study, a comparative evaluation of oocyte maturation media was performed: based on TCM 199 (Minitube) and Universal (Origio) culture media. At the second stage, the protocols for culturing bovine embryos were compared: Minitube and the two-stage Origio culture protocol with changing media. Therewith, it was found that the use of TCM 199 medium for oocyte maturation is more effective compared to Universal. Thus, at 48 hours of cultivation (the initial stage of embryo development), 64.3 ± 1.0 and 60.3 ± 1.4% of 2-8 cell embryos were obtained, and on Day 8 – 25.3 ± 1.0 and 20.0 ± 0.6% of blastocysts, respectively. The results of a comparison of bovine embryo culture protocols showed that when using both Minitube and Origio media, the percentage of division and the percentage of resulting embryos corresponded to their known values. It was found that the Minitube cultivation protocol is more effective than Origio. At 48 hours, the number of embryos obtained using the Minitube culture protocol was 1.3% higher compared to Origio, on Day 6 – by 7.8%, and on Day 8 – by 3.8%. The results obtained are a necessary component of the development of successful processes to produce bovine embryos *in vitro* with further implementation in the ruminant reproduction biotechnology

Keywords: embryonic development, Minitube and Origio culture media, cultivation protocols, ruminant reproduction biotechnology

Introduction

Animal husbandry plays an important role in agriculture and the economy of Ukraine. It provides profit and work for producers and other individuals working in value chains [1]. Currently, there is a well-known forecast that the world's population will exceed 9.2 billion by 2050, and this in turn will require an increase in food production by 50% [2]. However, it is impossible to achieve the required production volumes using conventional methods of animal reproduction biotechnology. To create volumes sufficient to meet the needs of the constantly growing population, the livestock industry needs to use modern methods of reproduction biotechnology, which contribute to obtaining greater efficiency from livestock productivity. Thus, genetic advance in dairy cattle breeding leads to an increase in the

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availability of milk, which provides nutrients to a growing population, without requiring an increase in the total number of animals that produce milk.

Programs to improve the genetic merits of the herd based on breeding are used to reproduce cows for many generations. However, the rate of change in individual traits as a result of breeding is relatively low (0.5-3.0% per year) [3]. At the same time, reproductive biotechnology for inducing changes can occur at an accelerated rate by increasing the intensity of selection without limiting the rate of reproduction.

A heifer is normally born with 10,000-35,000 healthy follicles and ova in the ovaries [4], but a typical beef cow will have no more than 10 calves in its lifetime, and dairy cows have even lower productivity. Scientific and biotechnological advances in animal reproduction in recent decades have led to the development of various methods, commonly referred to as assisted reproductive technologies. Their main task is to increase the offspring of genetically valuable animals and spread them all over the world [5]. When using these technologies, the average number of calves from a donor cow increases from 1 to more than 10 per year, which ensures an increase in reproduction rates and an increase in the genetic progress of the herd [6]. Additionally, it is possible to combine the selection of not only females, but also males according to the productivity of daughters. Thus, Rutledge [7] offers support for a herd of F1 cattle without the need to transport many pure-bred animals and apply further crossbreeding schemes using artificial insemination protocols *in vitro*. Such protocols allow fertilizing a cow's ova with the sperm of several bulls, effectively allowing the offspring of different producers to be born from the same cow within a year. It is also possible to implement the opposite scheme, when one sperm straw is used to fertilize oocytes obtained from several distinct donor cows, which allows the maximum use of valuable sperm samples [3].

Notably, the reproduction biotechnology in cattle breeding has been developing for a long time [8]. Thus, the first superovulations in cattle were implemented by Casida *et al*. as early as 1943 [9]. In the 1950s, Rowson performed the first successful transfer of embryos into cows in Cambridge, and the first commercial embryo transfer was performed as early as 1972 [10]. Successful *in vitro* fertilization in cattle was first reported in 1977 by Iritani and Niwa using sperm that had been capacitated in the oviduct or uterus of cows during oestrus or in the uterus of rabbits [11]. The birth of a calf after *in vitro* fertilization first took place in 1981 at the University of Pennsylvania [12].

Presently, the commercial transfer of bovine embryos is a significant international business, and most bovine embryos in the world are produced by *in vitro* fertilization technology [13]. Even though the methods used in the biotechnology of reproduction of cows are sufficiently studied, there are opportunities for their perfection. Therefore, scientists around the world continue to work on improving the protocols of *in vitro* fertilization of cattle.

The purpose of this study was to analyse the effect of two commercial panels of products – Origio (Origio, Denmark) and Minitube (Germany) on the effectiveness of oocyte maturation and development of bovine embryos *in vitro*.

Literature Review

Embryo production *in vitro* is rapidly developing because it has a great potential for strengthening genetic selection, increasing fertilization and optimizing crossbreeding schemes in beef and dairy cattle production systems [14]. According to Parrish *et al*. [15] in 1986, embryos that were obtained outside the body accounted for only 30% of the total number of cow embryos transferred worldwide. Statistics compiled by the International Society for Embryo Transplantation in 2011 showed that 732,862 embryos that were suitable for transfer were obtained by superovulation, and 572,342 embryos were obtained *in vivo* and transferred worldwide [16]. Moreover, of the latter, Europe, and North America account for 19% and 43% of their total number, respectively [16]. And in 2017, statistics showed that the number of IETS (International Embryo Technology Society) embryos, which were created *in vitro* in the entire world, was greater, compared to MOET programs (Multiple Ovulation Embryo Transfer – programs where embryos are washed from bovine uterine horns) [17].

However, despite the large volume of production of bovine embryos *in vitro*, the percentage ratio of the number of embryos suitable for transplantation to the number of used ova stays quite low. Thus, Lonergan and Fair indicate that only 30-35% of fertilized ova can reach a certain stage of development or meet high quality when their transfer to recipient cows is successful [18]. Similar data are highlighted by Ferré *et al*. [19], who claim that on Day 7 of cultivation, only 20-40% of probable zygotes reach the blastocyst stage.

Optimization of cultivation parameters *in vitro* allows increasing the percentage of embryos suitable for transplantation. That is why scientists around the world are working to solve this issue and develop various methodological approaches. The *in vitro* cultivation conditions to produce bovine embryos are aimed at maximally imitating the specific features of the oviduct to successfully manage this process outside the body [20]. This led to the creation of distinct types of culture systems using standard media, conditioned media [21], or by adding liquid from oviducts obtained during oestrus [22]. Goovaerts *et al*. [23] co-cultured zygotes with culture of cumulus cells, Chen *et al*. [24] – with the culture of epithelial cells of the oviducts of mammals, Ferraz *et al*. [25] – with oviduct cell culture on a chip. Rizos *et al*. [26] investigated the effect of serum on embryo development and cryotolerance, claiming that its absence has a positive effect. In turn, Leivas *et al*. [27] and Soto-Moreno *et al*. [28] indicate a positive effect of fetal bovine serum on the development of bovine embryos *in vitro*. Zullo *et al*. [29] claim that crocetin increases the quality and quantity of blastocysts from cows under *in vitro culture conditions*. As the above information suggests, there is presently significant information on the cultivation of bovine embryos, but most of these data differ from each other.

However, along with the development of innovative approaches to the cultivation of bovine embryos and changes in the composition of the culture medium, commercial environments are also being improved [30]. Comparing the effectiveness of the latter and determining the best one is a necessary component of developing successful protocols for obtaining embryos *in vitro*, with subsequent implementation in production.

Materials and Methods

The study was conducted during 2020-2022 based on the Educational and Scientific Laboratory "Centre for Animal Reproductology with Sperm and Embryo Bank" of the National University of Life and Natural Sciences of Ukraine. Ovaries from clinically healthy cows were selected in slaughterhouses and delivered to the laboratory in a thermos at 30-33°C no more than 3 hours after sampling. In the laboratory, the ovaries were washed 4 times in a sterile phosphate-salt solution of Dulbecco (Sigma, USA) with the addition of 0.075 mg/cm³ kanamycin sulphate (Sigma, USA) (solution temperature 37-38°C). The cumulus cell-oocyte complexes from the antral follicles (2-8 mm in size) of the ovaries of cows were removed in a laminar box by dissecting the follicles with a safety razor blade in an oocyte collection medium including 5 cm³ TL HEPES (Minitube, Germany) with the addition 30 mg of bovine serum albumin (Sigma, USA). Removal of cumulus cell-oocyte complexes, their selection and setting for maturation, fertilization, and subsequent cultivation were also performed in a sterile box (Fig. 1).

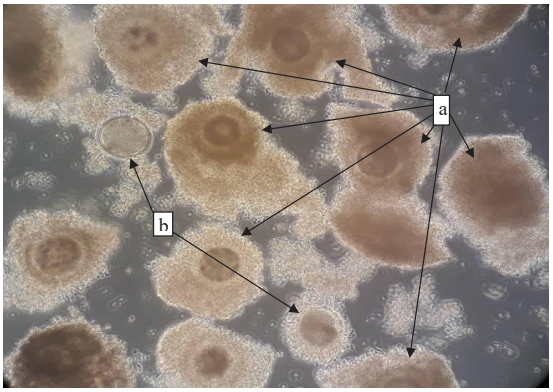

Figure 1. Cumulus cell-oocyte complexes of cows

Note: a – suitable for further cultivation; b – subject to culling. Native preparation. Mag.: ×80

After evaluation under a stereomicroscope SZ51 (Olympus, Japan), cumulus cell-oocyte complexes (COCs) of 120-130 μm with solid dense cumulus, intact transparent shell and homogeneous unvacuolized ooplasm of regular rounded shape were selected, without visible morphological signs of atresia (see Fig. 1).

The extracted COCs were washed 6 times in an oocyte collection medium. These complexes were selected and washed on a heating table at 37°C.

Bull sperm was prepared for fertilization using density gradients "Origio Gradient Series" (Origio, Denmark) and sperm capacitation medium (Minitube, Germany). The components of the gradient were heated to room temperature (20-25°C), all other reagents were equilibrated in a CO_2 incubator at 38.5-39.0°C and 5% CO_2 for at least 2 hours. The gradient was prepared by carefully layering on 1 cm³ of "Origio Gradient 40" and on 1 cm³ of "Origio Gradient 80", after which sperm previously thawed in a water bath was carefully introduced. The resulting system was centrifuged at a centrifugal force of 300 g for 20 minutes. The supernatant was removed, and the precipitate was transferred with a new sterile tip to a 2 cm³ tube media for preparation and capacitation of spermatozoa. The specified medium consisted of 5 cm³ base capacitation solution (Minitube, Germany), 30 mg bovine serum albumin (Sigma, USA), 0.55 mg sodium pyruvate (Sigma, USA) and 50 mm³ antibiotic-antimycotic (Sigma, USA). Then the contents of the test tube were centrifuged at a centrifugal force of 300 g for 5 minutes. Most of the supernatant was removed, and the procedure was repeated twice. After washing, the sediment was transferred to the bottom of a test tube with 1 cm³ of a new portion of medium for the preparation and capacitation of spermatozoa. The mobile sperm fraction was obtained using the swim-up method described by Parrish *et al*. [15]. Incubation for 1 hour was sufficient for mobile spermatozoa to rise to the upper layers of the medium, while dead and pathologically altered stayed at the bottom of the test tube. Motile spermatozoa were capacitated in the medium for preparation and capacitation for 4 hours of exposure to heparin (Sigma, USA) at a concentration of 20 μg/cm³ in a CO_2 incubator at 38.5-39.0°C and 5% CO_2. After capacitation, the spermatozoa were centrifuged at a centrifugal force of 200 g for 5 minutes. The supernatant was removed and 1 cm³ of fertilization medium was added, and the concentration of spermatozoa was counted in the Goryaev chamber.

At the first stage of the study, two media for oocyte maturation were compared:

1. The first medium included 4.5 cm³ of initial solution of the maturation medium TCM 199 (Minitube, Germany), 0.5 cm³ of cow oestrus serum, 0.125 IU of follicle-stimulating hormone (FSH) and 0.125 IU of luteotrophic hormone (LH) (50 mm³ of "Pluset", Laboratories Calier S.A., Spain), 0.125 IU of FSH ("FSH-Super", Agrobiomed, RF) and 50 mm³ of antibiotic-antimycotic (Sigma, USA).

2. The second medium included 4.5 cm³ of Universal (Origio, Denmark), 0.5 cm³ of cow oestral serum, 0.125 IU of FSH and 0.125 IU of LH (50 mm³ "Pluset", Laboratories Calier S.A., Spain) and 0.125 IU of FSH ("FSH-Super", Agrobiomed, Russia) and 50 mm³ of antibiotic-antimycotic (Sigma, USA).

Oocytes were matured *in vitro* for 22-24 hours in 4-well plates (Oosafe, USA). 300 mm³ of medium was introduced into each well, covered with mineral oil (Origio, Denmark), 25 COCs were added and cultivated in a CO_2 incubator at 38.5°C and 5% CO_2. After cultivation, the oocytes were co-cultured with spermatozoa. The specified medium included 5 cm³ base capacitation solution (Minitube,

Germany), 30 mg of bovine serum albumin, 0.11 μg of sodium pyruvate, 0.2 mg of heparin, and 50 mm^3 of antibiotic-antimycotic (Sigma, USA). Oocytes were fertilized in 4-well plates (Oosafe, USA). 300 mm^3 of the medium was covered with mineral oil (Origio, Denmark) for 18 h after the addition of capacitated spermatozoa (at the rate of 1×10^6 motile spermatozoa/cm^3). The number of oocytes in the well ranged from 5 to 10. After co-culture, bovine oocytes with loose enlarged cumulus were released from cumulus cells by gentle pipetting in 0.1% hyaluronidase solution (Sigma, USA). Then the oocytes were washed from the enzyme in 5-6 drops of TL HEPES (Minitube, Germany) and transferred to a culture medium comprising 5 cm^3 of culture medium with pyruvate (Minitube, Germany), 0.5 cm^3 of bovine estrous serum, 200 mm^3 of essential amino acids (Sigma, USA), 50 mm^3 of substitute amino acids and 50 mm^3 of antibiotic-antimycotic (Sigma, USA). Fertilized oocytes were discovered by division from the 2- to 8-cell stage 48 hours after contact with spermatozoa. On Day 7 of cultivation, the resulting blastocysts were evaluated under a stereomicroscope and their percentage was determined.

Since TCM 199 maturation medium turned out to be more effective, further studies were conducted using it.

At the second stage of the study, two protocols for culturing bovine embryos were compared:

1. Minitube cultivation protocol (n = 50):

– oocytes were co-cultured with spermatozoa for 18 h in a fertilization medium including 5 cm^3 of base capacitation solution (Minitube, Germany), 30 mg of bovine serum albumin, 0.11 μg of sodium pyruvate, 0.2 mg of heparin, and 50 mm^3 of antibiotic-antimycotic (Sigma, USA). Subsequently, the embryos were transferred to the embryo culture medium;

– the medium for embryo cultivation included 5 cm^3 of culture medium with pyruvate (Minitube, Germany), 0.5 cm^3 of bovine oestrous serum, 200 mm^3 of essential amino acids (Sigma, USA), 50 mm^3 of substitute amino acids and 50 mm^3 of antibiotic-antimycotic (Sigma, USA), where they were cultured for 8 days.

2. Two-stage Origio cultivation protocol (n = 50) with changing media:

– oocytes were co-cultivated with spermatozoa for 18 hours in Sequential Fert medium (Origio, Denmark) with the addition of 10 mm^3/cm^3 of an antibiotic-antimycotic (Sigma, USA), after which the selected embryos were transferred to the medium for their cultivation;

– in Sequential Cleav medium (Origio, Denmark) with the addition of 10 mm^3/cm^3 antibiotic-antimycotic (Sigma, USA), embryos were cultured for 48 hours until their 4-8-cell stage;

– in Sequential Blast medium (Origio, Denmark) with the addition of 10 mm^3/cm^3 of an antibiotic-antimycotic (Sigma, USA), the embryos were further cultured for up to 8 days.

Embryos were cultivated in microdrops of 65 mm^3 (3 embryos per drop) under a layer of mineral oil (Origio, Denmark) in culture dishes (Oosafe, USA) in a CO_2 incubator at 38.5°C with 5% CO_2 and 5% O_2.

The effectiveness of diverse bovine embryo culture protocols was evaluated on days 6 and 8. Therewith, the percentage of blastocysts obtained was found under a stereomicroscope.

Statistical processing of the obtained experimental results was performed according to N.A. Plokhinsky [31] and E.V. Montsevichyute-Eringeme [32] using the Microsoft Excel data analysis package [33]. Arithmetic mean values and their errors were found, and the probability of difference between parallel data sets was determined. In all cases, the difference was considered reliable at $P < 0.05$.

Results and Discussion

Maturation of ova outside the body or *in vitro* Maturation (IVM) is an important stage of the technology of obtaining bovine embryos *in vitro*. Its improvement will considerably increase the efficiency of *in vitro* fertilization and, as a result, increase the percentage ratio of the number of embryos suitable for transplantation to the number of ova used. Along with the bovine-adapted maturing medium TCM 199 (with the addition of bovine oestrous serum, FSH and LH), this study presents the effectiveness of the Universal medium (with the addition of bovine oestrous serum, FSH and LH in the same concentration), which is normally used in humanitarian medicine (Table 1).

Table 1. The influence of different environments on the maturation and further development of oocytes *in vitro*, M ± m, n = 3

No. seq.	Composition of the medium for oocyte maturation	Number of COCs, pcs	Number of expertiments	Division, %	Development to the blastocyst stage (Day 8 of cultivation)	
					% of the number of oocytes	% of the number of embryos
1	4.5 cm^3 of the initial solution of maturation medium TCM 199, 0.5 cm^3 of bovine oestrous serum, 0.25 IU FSH and 0.125 IU LH	100	3	64.3 ± 1.0	25.3 ± 1.0	38.8 ± 2.1
2	4.5 cm^3 of Universal, 0.5 cm^3 of bovine oestrous serum, 0.25 IU FSH and 0.125 IU LH	100	3	60.3 ± 1.4	20.0 ± 0.6**	33.2 ± 0.7*

Note: * $P < 0.05$, * * $p < 0.01$, compared to the indicators of medium No. 1

A comparison of the two maturation media, TCM 199 and Universal, did not reveal a statistically significant difference in the percentage of embryos at the stage of 2-8 cells at 48 hours of cultivation, although it was 4% higher when using medium No. 1 compared to medium No. 2. At the same time, on Day 8 of oocyte culture, a significant difference ($P < 0.01$) between the groups was established. Thus, in the group of dishes, the basis of which medium for maturation was TCM 199, the number of blastocysts was 25.3 ± 1.0% per 100 taken COCs. When using a medium for

oocyte maturation, which included Universal, the indicator was lower by 5.3% ($P < 0.01$) compared to the indicators of medium No. 1 (per 100 COCs).

The above pattern was also observed when calculating the percentage of blastocysts from the number of embryos. Thus, on Day 8 of cultivation, it was noted that the values of this indicator in Group No. 2 decreased by 5.6% ($P < 0.05$) compared to Group No. 1. Analysing the data obtained, the use of TCM 199 medium for maturation of oocytes is more effective compared to Universal both at the initial stages of embryo development – division, and at the following stages – up to the blastocyst stage.

Syngina *et al*. consider 30.6% the best result in obtaining blastocysts [34]. Inaba *et al*. indicate that the percentage of blastocysts obtained is also substantially affected by the sperm used for fertilization. Therefore, these indicators in their study ranged within 22-44% [35]. Sirard *et al*. [36] reported that culturing oocytes in medium containing TCM 199 supplemented with 10% heat-treated fetal calf serum, FSH (0.5 μg/cm^3), LH (5 μg/cm^3), and estradiol-17beta (1 μg/cm^3) is the most effective of those studied because the frequency of obtaining late morulae or blastocysts was 28%. According to the research results, when using both TCM 199 and Universal as a medium for oocyte maturation (which were not previously used for bovine oocyte maturation), the percentage of divisieon and the percentage of embryos obtained were within the data presented by other scientists [34-36].

Presently, the production of embryos *in vitro* has become the dominant method of producing bovine embryos for transplantation to female recipients. However, the protocols to produce embryos *in vitro* are not agreed upon. Thus, the proportion of embryos developing to the blastocyst stage is usually less than 40%. While embryos developing *in vivo* become blastocysts in 50-80% [37].

One of the strategies for improving *in vitro* embryo production programs is to change the cultivation conditions, which will stimulate development and improve the ability of embryos to implant. There is evidence [38] that changes in the culture medium can lead to the loss of important regulatory factors for embryos. It is known that changes in the cultivation environment do not lead to the accumulation of toxic substances [38].

A comparative evaluation of the cultivation protocols of two different producers was performed: Minitube, which does not involve changing the environment during cultivation, and Origio with a two-stage cultivation protocol with changing "Sequential Cleav" to "Sequential Blast" for 48 hours of incubation.

TCM 199 medium was used for oocyte maturation because according to the results of the study given above, it turned out to be the most effective. After purification from cumulus (Fig. 2), oocytes were transferred to the Minitube culture medium using the first protocol and "Sequential Cleav" – using the second protocol. At 48 hours, the first differences in embryo development were observed, which lasted up to Days 6 and 8 (Table 2).

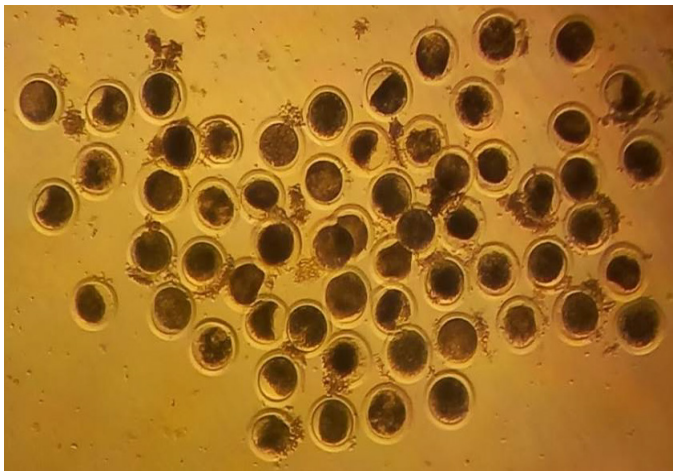

Figure 2. Bovine oocytes after co-cultivation with spermatozoa and purification from cumulus cells. Native preparation. Mag.: ×80

Table 2. Comparative evaluation of embryo culture protocols and their further development *in vitro*, M ± m, n = 3

No. seq.	Bovine embryo cultivation protocol	Number of COCs, pcs	Number of expertiments	Division, %	Development to the blastocyst stage, % of the number of embryos	
					(Day 6)	(Day 8)
1	**Minitube** (medium for fertilization (18 hours) and medium for cultivation (up to 8 days)	100	3	64.0 ± 1.7	35.9 ± 2.1*	38.8 ± 0.8*
2	**Origio** ("Sequential Fert" (18 hours), "Sequential Cleav" (48 hours) and "Sequential Blast" (up to 8 days)	100	3	62.7 ± 1.5	28.1 ± 1.8	35.0 ± 1.1

Note: * $P < 0.05$, compared to the indicators of Protocol No. 2

The results presented in Table 2 suggest that using the first protocol of cultivation of 2-8 cell embryos was 1.3% more compared to Protocol No. 2 (Origio scheme). Therewith, there is no statistically significant difference between the indicators.

On Day 6 of cultivation, 7.8% more blastocysts were obtained from the number of embryos using Protocol No. 1 compared to Protocol No. 2. On Day 8 of cultivation, 3.8% more blastocysts were observed from the total number of embryos using Protocol No. 1 compared to Protocol No. 2.

Thus, on Day 8 of cultivation, an average of 25 blastocysts per 100 COCs were obtained using the Minitube protocol. The use of Origio media resulted in 23 blastocysts. The results show that the Minitube protocol is significantly more effective than Origio for the cultivation of bovine embryos.

In the embryo, the number of cells and their placement in the blastocyst are considered indicators of the embryo's developmental potential [39-41]. That is why the described studies found the percentage of blastocysts from the number of embryos on Days 6 and 8 of cultivation.

Thus, on Day 6 of cultivation, it was found that the embryos are at various stages of development (Fig. 3).

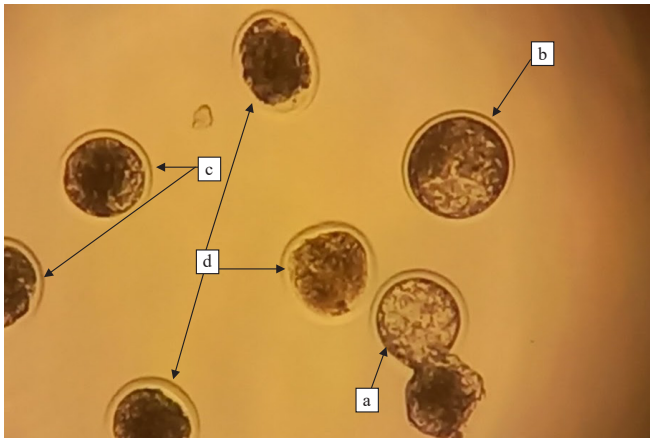

Figure 3. Bovine embryos at various stages of development (Day 6 of cultivation)

Note: a) blastocyst emerging from the zona pellucida; b) expanded blastocyst; c) early blastocyst; d) morula. Native preparation. Mag.: ×200

Thus, Oliveira *et al*. [42], investigating the kinetic laws of success and the reasons for the inability of embryos to reach the blastocyst stage, obtained the maximum percentage of blastocyst yield – 29.9%. While in Stoliarova and Leontieva [43], using KSOM medium with the addition of 1 mM glutamine and bovine serum albumin at a concentration of 1 mg/cm^3, this indicator was 22.3%. According to the study results, the use of the bovine embryo cultivation protocol, both Minitube and Origio, which was not previously used for the cultivation of bovine embryos, the percentage of division and the percentage of obtained embryos were within the data presented by other scientists [42; 43].

Conclusions

Recent advances in assisted reproductive technologies are a powerful tool that can be used to improve and address the challenges of animal husbandry in the future. Thus, as a result of using the Minitube maturation medium for 48 hours of cultivation, 4% more embryos were obtained at the 2-8 cell stage, compared to Universal, which is 64.3 ± 1.0 and 60.3 ± 1.4%, respectively. On Day 8 of cultivation, when using the TCM 199 oocyte maturation medium, a larger number of blastocysts per 100 cumulus cell-oocyte complexes was observed compared to Universal, namely: 25.3 ± 1.0 and 20.0 ± 0.6, respectively. At 48 hours of cultivation using the Minitube protocol, 1.3% more 2-8 cell embryos were obtained compared to the Origio scheme. Therewith, the specified indicator was 64.0 ± 1.7 and 62.7 ± 1.5%, respectively. On Day 6 of cultivation, there are 7.8% more blastocysts from the number of embryos using the Minitube culture protocol compared to the Origio protocol. Using the Minitube bovine embryo culture protocol, 38.8 ± 0.8 blastocysts were obtained on Day 8 of the study, which is 3.8% more than Origio.

Applying these results to ruminant reproduction biotechnology will help manage the limited availability of resources and the increased need for food production because producers will be able to cause rapid genetic changes to create the next generation of animals.

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Вплив різних середовищ на дозрівання ооцитів та розвиток ембріонів великої рогатої худоби в умовах *in vitro*

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Анотація. Згідно з даними International Embryo Technology Society, кількість ембріонів великої рогатої худоби, які виробляються за технологією запліднення в умовах in vitro, зростає з кожним роком. Однак, незважаючи на великі обсяги їх виробництва, ефективність цього методу залишається низькою і потребує удосконалення. Тому, метою дослідження було порівняти ефективність двох комерційних середовищ – Origio Sequential Series (Origio, Данія) і панелі продуктів виробництва Minitube (Німеччина) щодо дозрівання ооцитів та розвитку ембріонів великої рогатої худоби в умовах *in vitro*. На першому етапі дослідження проведено порівняльну оцінку середовищ для дозрівання ооцитів: на основі культуральних середовищ ТСМ 199 (Minitube) та «Universal» (Origio). На другому – здійснено порівняння протоколів культивування ембріонів великої рогатої худоби: Minitube та двоступеневого протоколу культивування Origio зі зміною середовищ. При цьому встановлено, що використання середовища ТСМ 199 для дозрівання ооцитів є ефективнішим у порівнянні з «Universal». Так, на 48 год культивування (початковий етап розвитку ембріона) отримано 64,3 ± 1,0 і 60,3 ± 1,4 % 2-8 клітинних ембріонів, а на 8 добу – 25,3 ± 1,0 і 20,0 ± 0,6 % бластоцист, відповідно. Результати порівняння протоколів культивування ембріонів великої рогатої худоби показали, що за використання середовищ як Minitube, так і Origio відсоток ділення та відсоток отриманих ембріонів відповідали їх відомим значенням. При цьому, встановлено, що протокол культивування Minitube є ефективнішим за Origio. Зокрема, на 48 год кількість отриманих ембріонів за використання протоколу культивування Minitube була на 1,3 % більшою порівняно з Origio, на 6 добу – на 7,8 %, а на 8 добу – на 3,8 %. Отримані результати є необхідною складовою розробки успішних процесів виробництва ембріонів великої рогатої худоби в умовах in vitro з подальшим впровадженням у біотехнологію відтворення жуйних

Ключові слова: ембріональний розвиток, культуральні середовища Minitube і Origio, протоколи культивування, біотехнологія відтворення жуйних

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Effectiveness of Detergents and Disinfectants in the Production of Cow's Milk

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Abstract. The relevance of this study is conditioned upon the need to improve the hygiene of milking cows by selecting detergents and disinfectants with different component compositions that ensure the quality and safety of dairy products. The purpose of this paper was to investigate the effectiveness of solutions of modern chlorine-containing and chlorine-free detergents and disinfectants on the test cultures of microorganisms, the sanitary and hygienic condition of the milking parlour, parts of milking equipment, and the skin of the udder teats of cows. The total number of bacteria was determined according to the requirements of DSTU ISO 15214:2007, by inoculating 1 cm^3 of the test material on meat-peptone agar followed by incubation at an average temperature of 36°C for 24-48 h. Cultivation was performed in a thermostat at 37°C. After incubation, colonies of grown microorganisms were counted, and the number of colony-forming units was determined. Detergents and disinfectants "Chloramine B", "Sanalcalin" and "Neochlor" have a detrimental effect on the growth of test cultures of *E. coli, S. aureus* and *P. aeruginosa*. "Desmol" suppresses the growth of test cultures of *E. coli* and *P. aeruginosa* after 20 minutes. The number of colonies of microorganisms in the air of the milking parlour is minimal at the beginning and greatest at the end of milking. 0.5% solution has a detrimental effect on *E. coli* and *S. aureus*, and slightly affects the growth of *Salmonella spp*. At the same time, this product is effective against microbial contamination of the skin of the udder teats of cows for its use in pre-milking disinfection. During the treatment of milking equipment with a 0.5% "Gralan Gel" solution, the total bacterial inoculation of the milking gum is reduced by 94.1%, the collector – by 98.4%, the milk hose – by 96.5%, the tank of the milking machine – by 97.4% compared to the indicators before processing the milking equipment with detergents and disinfectants. According to the results of the study, the best efficiency in reducing the general bacterial contamination of milking equipment, the harmful effect on opportunistic microflora and microbial contamination of the skin of the udders of cows when used in pre-milking disinfection at milk production enterprises shows a 0.5% solution of the alkaline detergent and disinfectant "Gralan Gel". Thus, it is advisable to use a 0.5% solution of alkaline detergent and disinfectant "Gralan Gel" for producers of cow's raw milk to reduce the total bacterial contamination of milking equipment and cow udders

Keywords: industrial cattle breeding, disinfection, bactericidal properties, microbial contamination

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Introduction

Providing the population with high-quality food products, including milk and dairy products, is one of the principal tasks in solving the food problem in the country. Presently, modern dairy complexes use intensive integrated technologies in compliance with hygienic and veterinary and sanitary requirements for farms. However, a considerable part of farms still uses partially converted cowsheds and auxiliary production facilities that meet the standards of the 1960s and 1970s. In such farms, the most common is the use of milking equipment such as "Maiga" and others, when portable milking machines are used, and conditions are created that contribute to the development of saprophytic, and sometimes pathogenic microorganisms. These conditions include elevated temperature, humidity, significant dust content in the air, insufficient ultraviolet radiation, and crowding of animals.

The number of microorganisms in the air of premises for cattle varies significantly – from 12 to 100 thousand microbial bodies per 1 m^3. Therewith, animals are significant sources of bioaerosols. Thus, among the thirty-four isolated strains of staphylococcus, *S. equorum* (35%), *S. succinus* (26%) and *S. xylosus* (15%) were the most common and resistant to macrolides (erythromycin), lincosamides (clindamycin), and methicillin. Therefore, it is necessary to support proper sanitary conditions and hygiene of premises for keeping animals [1].

Furthermore, the intensification of animal husbandry, the transfer to an industrial basis due to the large concentration of cows on dairy farms led to hypodynamia, metabolic disorders, and an increase in cases of mastitis. The intensification of production and the widespread use of machine-type cow milking has led to the spread of this disease in all countries of the world, and a decrease in the quality of milk [2]. Therewith, the quality of milk and dairy products depends on the hygienic conditions of keeping lactating animals [3].

One of the principal requirements listed in regulatory documents is the requirements for air hygiene of livestock premises where lactating animals are constantly located, and with which they are in constant contact. At the same time, saprophytic and sometimes pathogenic microorganisms have the main influence on the living organism of the polluted air of livestock premises. Pathogens of many diseases are spread by air, predominantly by its convection currents. The aerogenic route of disease transmission becomes essential in conditions of high concentrations of animals [4].

Therefore, adequate management of the dangerous factor that can appear in food products, namely milk, at any link in the food chain, is an important task for producers. To solve this, the International Organization for Standardization developed ISO 22000:2005 "Food safety management systems. Requirements for any organizations of the food chain", which has been in force in Ukraine since April 2007, as DSTU ISO 22000:2007 [5].

Thus, obtaining milk of high sanitary quality is of great social importance. Over the past 5 years, considerable attention has been paid to udder sanitation, which substantially affects the bacterial contamination of milk and contributes to improving its quality and safety. Therewith, maintaining the hygiene of the udder of cows, which includes cleaning, washing, and disinfection, primarily aimed at reducing the risk of the disease [6].

Several disinfectants are currently recommended for sanitary treatment of the udder. These are preparations of iodine monochloride, chlorine, Desmol, Vesan, calcium hypochlorite, quaternary ammonium salts, an aqueous suspension of hexochlorophene and triethanolamine, and an antiseptic emulsion [7; 8]. When choosing a disinfectant or a method of disinfection, it is necessary to consider that its disinfection effect depends on several factors, namely: the degree of endurance of microorganisms; concentration of the solution; solution temperature; pH of the medium; the physical and chemical nature of the object of disinfection; spectrum of antimicrobial action. Today, based on their effectiveness in reducing the total bacterial contamination of milk, combined detergent-disinfectants are most often used, which include: "Desmol", "Chloramine B", "Sanalcalin", "Neochlor" and "Gralan Gel".

The purpose of this study is to investigate the bactericidal properties of chlorine – containing detergents and disinfectants: "Desmol", "Chloramine B", "Sanalcalin", "Neochlor", to assess microbial contamination of the milking parlour, the skin of the udder teats of cows and the rubber of milking cups, as well as the general bacterial contamination of parts of milking equipment during the production of cow milk using 0.5% solutions of soda ash, "Desmol" and alkaline detergent and disinfectant "Gralan Gel".

Literature Review

Recently, milk producers have been using environmentally friendly products. During their application, the antimicrobial component (ozone) is created from atmospheric oxygen and quickly disintegrates with the end of the disinfection event, without polluting the object and atmosphere with residual products. That is why ozonation in comparison with traditional disinfection methods can substantially reduce the consumption of biologically pure water, energy, as well as the costs associated with transportation and storage of disinfectant [9].

One of the reasons for the deterioration of milk quality and safety is the disease of cows with subclinical mastitis. Subclinical mastitis is infectious in nature, although the trigger in the disease is an economic cause. Microflora is one of the main and principal factors in subclinical mastitis in cows. Due to the contamination of milk with pathogenic microorganisms, it can cause human diseases. The causative agents of subclinical mastitis are more often pathogenic forms of cocci, which are found both in the external environment and on the skin of the udder of animals. When milking cows, milking machines, with any milking system, are used without disinfection. If a cow with an infected udder enters the milking process, direct infection from one animal to another occurs. Therefore, subclinical mastitis is widespread in the herd. To break this pathogenic chain, it is necessary to conduct sanitary and hygienic measures, one of which is disinfection [10; 11].

Currently, several disinfectants of domestic and foreign production are used in the veterinary practice of Ukraine. Most of them do not fully meet the requirements of versatility, stability during transportation, degree of dissolution in water, bactericidal action, and safety for living

organisms. Therefore, there is a need to develop other combined-acting disinfectants. A mandatory condition for the introduction of a disinfectant in Ukraine is practical testing, during which the disinfectant in real conditions of use has the possibility of contact with populations of microorganisms [12].

A promising area in veterinary medicine is the creation of new and improvement of available disinfectants. The purpose of their creation is to expand the spectrum of antimicrobial activity and the ability to counteract the formation of resistant strains of microorganisms. The effect of such disinfectants should be harmful to viruses and fungi and environmentally safe. One of these means is an ozone-air mixture – the use of ozone technologies is an alternative to conventional disinfectants and antibacterial agents [13].

Currently, in Ukraine, the assessment of the quality of disinfection includes visual control of the sanitary condition and bacteriological control. Bacteriological control is exercised by specialists of accredited laboratories, by sampling flushes from livestock facilities and evaluating the hygienic condition according to the indicators of general bacterial contamination, colour titre, and the presence of pathogenic and conditionally pathogenic microflora [14].

Thus, the main factor in the system of ensuring food safety and quality is the sanitary preparation of animals, premises, and equipment for obtaining high-quality milk.

Materials and Methods

The research was conducted in 2021 at the I.S. Zagaievskyi Department of Veterinary and Sanitary Expertise, Hygiene of Livestock Products and Anatomical Pathology of the Bila Tserkva National Agrarian University, Laboratories of Svitlovodsk and Bashtanka creameries of the Dnipropetrovsk region. Scientific and economic experiments were performed in the following milk production farms: Agrofirma Piatykhatska LLC, Progres LLC, Zarichne LLC and Agrofirma Mariampolska LLC.

In experimental farms, the method of milking in portable milking machines is used. When determining the bactericidal properties of detergents and disinfectants, they were used at different concentrations and chlorine content: 0.25% and 0.5% solution – "Desmol" (chlorine content – 120, 250 mg/dm^3), 0.25% and 0.5% solutions – "Chloramine B" (chlorine content – 120 mg/dm^3, 250 and 500 mg/dm^3) and "Sanalcalin" (chlorine content – 250 and 500 mg/dm^3), and 0.25%, 0.5% and 0.7% solution – "Neochlor" (chlorine content – 250 and 500 mg/dm^3). Therewith, the temperature of these solutions is different: "Desmol" – 50 ± 5°C, "Chloramine B" – 16 ± 2°C and "Neochlor" – 16 ± 2°C. For these laboratory studies, test cultures of *E. coli* (strain No. 078), *S. aureus* (strain No. 209-P), and *P. aeruginosa* (strain No. 27.99) were used.

The antimicrobial effect of the alkaline detergent and disinfectant "Gralan Gel" (Germany) was investigated. This is a complex detergent and disinfectant that contains a complex of special protective components: glycerine, lanolin, allantoin, and other special additives. These components allow disinfecting any premises, as well as keeping the skin of the udder and teat soft, smooth, and prevent pathogens from entering the teat canal. The antimicrobial effect of the alkaline detergent and disinfectant "Gralan Gel" was evaluated by the effect on the dynamics of microbial air pollution in the milking unit, as well as in samples of flushes from the walls of the milking parlour, the skin of the udder teats of cows and from the rubber of milking cups. At the beginning of the study, after wet cleaning of the milking parlour, the room air was treated with a 0.5% solution of Gralan Gel. 15 minutes after airing the room, milking of cows began.

The study was conducted in three stages. At the first stage, the bactericidal properties of these detergents and disinfectants were investigated.

At the second stage, the dynamics of the general bacterial contamination of the milking parlour air, flushes from the walls of the milking parlour, from the rubber of milking cups and the skin of the udder teats of cows during the milking period (lasts 6 minutes) were established – at its beginning, in the middle (after 2-3 minutes), and at the end. The total number of bacteria was determined according to DSTU ISO 15214:2007 [15], by inoculating 1 cm^3 of the test material on meat-peptone agar (MPA) followed by incubation at 36 ± 2°C for 24-48 h. Cultivation was performed in a thermostat at 37°C. The nature of microflora growth on the media was determined after 24 and 48 hours [16-18].

The cultures of microorganisms isolated in washings from milking equipment were identified based on morphological, tinctorial, cultural, and biochemical data using Bergey's bacteria identifier [19; 20].

At the third stage, microbiological studies of flushes from milking equipment were performed using various detergents and disinfectants. For this purpose, after incubation, colonies of grown microorganisms were counted and the number of colony-forming units per unit volume of the test material (CFU/cm^3) was determined [17; 18].

Statistical processing of the obtained results was performed using the Microsoft Office Excel 2007 software and the Fischer-Student method. Therewith, the values of arithmetic mean values and their statistical error were considered. The probability of the difference between the obtained indicators, by comparing them, the arithmetic mean (M) and the standard error of the arithmetic mean (m) were determined. The difference between the values of indicators was considered statistically significant at a level of more than 95% ($P < 0.05$).

Results and Discussion

The results of the study of the bactericidal properties of detergents and disinfectants are presented in Table 1.

Table 1. Bactericidal properties of detergents and disinfectants, n = 5

Means, solution temperature	Solution concentration (%)	Active chlorine content (mg/l)	Exposure (min) test cultures								
			S. aureus			*E. coli*			*P. aeruginosa*		
			5	10	20	5	10	20	5	15	20
"Desmol", t 50 ± 5°C	0.25	120	+	+	+	+	+	+	+	+	+
	0.5	250	+	+	+	+	+	–	+	+	–

Table 1, Continued

Means, solution temperature	Solution concentration (%)	Active chlorine content (mg/l)	Exposure (min) test cultures								
			S. aureus			*E. coli*			*P. aeruginosa*		
			5	10	20	5	10	20	5	15	20
"Chloramine B", t 16 ± 2°C	0.25	120	+	–	–	–	–	–	–	–	–
	0.5	250	–	–	–	–	–	–	–	–	–
	0.7	500	–	–	–	–	–	–	–	–	–
"Sanalcalin", t 16 ± 2°C	0.25	250	–	–	–	–	–	–	–	–	–
	0.5	500	–	–	–	–	–	–	–	–	–
"Neochlor", t 16 ± 2°C	0.25	250	–	–	–	–	–	–	–	–	–
	0.5	500	–	–	–	–	–	–	–	–	–

Note: "+" – available growth, "–" – no growth

The results presented in Table 1 show that a 0.25% solution of the detergent and disinfectant "Desmol" with an active chlorine content of 120 mg/dm^3, with an exposure of 20 minutes, did not stop the growth of the test culture of *E. coli*, *S. aureus*, and *P. aeruginosa*. At the same time, when using a 0.5% "Desmol" solution with a chlorine content of up to 250 mg/l, no growth of the *E. coli* and *P. aeruginosa* test culture was detected 20 minutes in. Therewith, the test culture of *S. aureus* is less sensitive to this disinfectant. During the study, the disinfectant "Chloramine B" in all applied concentrations effectively acted on test cultures of microorganisms. However, the activity of this disinfectant against *S. aureus* was manifested only after 10 minutes at an active chlorine content of 120 mg/dm^3.

At the same time, 0.25 and 0.5% solutions of detergents and disinfectants – "Sanalcalin" and "Neochlor" with an active chlorine content of 250 and 500 mg/dm^3 of the solution had a detrimental effect on the growth of test cultures (Table 1). Thus, the test cultures of *S. aureus* turned out to be less sensitive to the effect of the tested detergents and disinfectants. In general, for the treatment of livestock premises, it is necessary to carefully select detergents and disinfectants according to their concentration and exposure. Other scientists have proven that microorganisms isolated from dairy equipment form high- and medium-density biofilms that protect them during disinfection of dairy equipment [6]. Furthermore, it is necessary to consider the sensitivity of microorganisms to different temperature ranges [7].

It is possible to obtain high-quality and safe milk only from healthy cows and in compliance with sanitary and hygienic requirements during its production. One of these requirements is to eliminate the possibility of bacterial contamination during milking. The main sources of contamination at the milking stage are the sanitary cleanliness of udders and dairy equipment. For sanitary treatment of milking equipment, chemical disinfectants are most often used in the form of solutions, emulsions, and suspensions of various concentrations and aerosols, and gases – for disinfection of dairy equipment and care items [6; 8]. At the same time, milk producers strive to reduce the number of chemical treatments, which is economically feasible, reduces risks to animal health and preserves the quality of products. Thus, when evaluating the functional state of the udder of cows, it is necessary to monitor the content of somatic cells in milk. Depending on the result, it becomes possible to adjust the frequency of treatment with detergents and disinfectants for both the udder and dairy equipment [21]. When milking cows kept in a stall/tie-up housing into portable milking buckets, it is difficult to obtain quality indicators of extra or higher-grade milk according to the requirements of the current national standard DSTU 3662:2018 "Raw cow milk. Technical conditions" [22].

Therefore, it is important to evaluate the sanitary condition of the milking parlour regarding the dynamics of general bacterial contamination during the milking period – at its beginning, in the middle, and at the end. It was found that at the beginning of milking, the number of colonies of microorganisms in the air of the milking parlour was insignificant (up to 70 thousand/m^3) (Fig. 1).

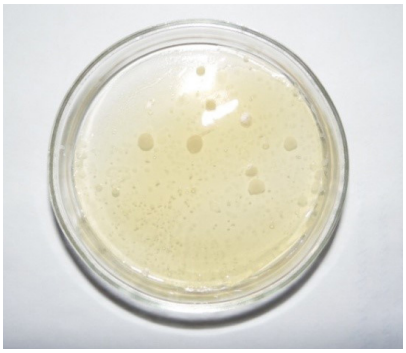

Figure 1. Total number of bacteria in the milking parlour air at the beginning of milking

Figure 2 shows that the number of colonies that have grown on the MPA increases to 235 thousand/m^3 in the middle of milking.

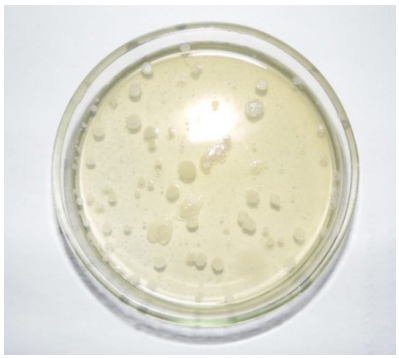

Figure 2. Total number of bacteria in the milking parlour air in the middle of milking

This is explained by the fact that cows are not sanitized before being moved to the milking parlour. The unsatisfactory condition of the skin of cows leads to the accumulation of microorganisms in the room, which can get on the dairy equipment, specifically in the milk glasses, and from there in the milk. Figure 3 shows that the number of colonies at the end of milking increased significantly (up to 485 thousand/m^3) compared to its beginning and middle.

Figure 3. Total number of bacteria in the milking parlour air at the end of milking

The fluctuation of the air leads to the fact that microorganisms, together with the air currents, fall onto the udder already prepared for milking and into the milking cups. And, as a result, they increase the bacterial contamination of milk by 1.5-2 times. Therefore, milking machines should be connected immediately after sanitary preparation of the udder for milking, and not after some time. Typically, especially with the method of tie-up housing and milking in portable milking machines, machine milking operators often wash the udders of several cows together, and only then connect the milking machine.

Such actions lead to the spread of microorganisms among animals. It is known that the lower the microbial contamination of milk, the longer the period when milk meets the requirements of the current national standard in terms of sanitary and hygienic indicators [22].

Therewith, the authors of this paper conducted a microbiological study of the sanitary condition of the milking parlour, the skin of the udder teats of cows and the rubber of milking cups during the entire milking period. The results are presented in Table 2.

Table 2. Dynamics of microbial contamination of samples without the use of detergents and disinfectants, n = 5

Sample		*E. coli*	*Salmonella spp.*	*S. aureus.*
Air in the milking unit	at the beginning of milking	+	–	–
	in the middle of milking	+	+/–	+/–
	at the end of milking	+	+	+
Flushes from the walls of the milking parlour	at the beginning of milking	+	–	–
	in the middle of milking	+	+	+
	at the end of milking	+	+	+
Washes from the skin of cows' udders	before washing	+	+	+
	after washing	+/–	–	+/–
Flushing from the rubber of milking cups	at the beginning of milking	+	–	–
	in the middle of milking	+	+	+
	at the end of milking	+	+	+

Based on the results presented in Table 2, it follows that the sanitary condition of the milking parlour at the beginning of milking and at the end has significant differences. In the air of the milking parlour at the beginning of milking, the growth of *E. coli* and in some cases *Salmonella spp*. was detected; on the other hand, the pathogen *S. aureus* was absent. However, in the middle of the milking period and at the end, the growth of *E. coli*, *Salmonella spp*. and *S. aureus* was found in the air of the milking parlour. Comparable results were obtained when finding the microbial contamination of flushes from the walls of the milking parlour. At the beginning of milking, only *E. coli* and *Salmonella spp*. and *S. aureus* were not detected, but later, in some samples, the growth of colonies of these pathogens appeared. After washing the udder and teats with water, the growth of *E. coli* was detected in some samples, and *S. aureus* or their association in some samples.

According to the study of the sanitary condition of the rubber of milking cups, it was found that at the beginning of milking it contained only *E. coli*, while *Salmonella spp*. and *S. aureus* were absent. However, in the middle of the milking period, *E. coli*, *Salmonella spp*., and *S. aureus* were detected.

Thus, the obtained results suggest that the sanitary condition of the milking parlour, even with satisfactory pre-milking preparation, changes substantially. The sanitary condition of this room is substantially affected by the cleanliness of the skin of animals, namely the udder, and the presence of mastitis. Thus, according to the data of other authors [23], 62.0% of cows are diagnosed with subclinical mastitis, and *S. aureus* is isolated from most milk samples examined for bacterial insemination. Therewith, scientists have proven that the risk factors are contaminated udders and limbs in cows, the lactation period, non-compliance with the requirements for the hygiene of the milking area and the lack of milking of the first portions of milk before milking.

The results of the study of the dynamics of microbial contamination of the selected air samples in the milking block, washings from the walls of the milking parlour, the skin of the milking cows, the udders of cows, and from the rubber of the milking cups with the use of the cleaning and disinfecting agent "Gralan Gel" are presented in Table 3.

Table 3. Dynamics of microbial contamination of samples with the use of detergent and disinfectant "Gralan Gel", n = 5

Sample		*E. coli*	*Salmonella spp.*	*S. aureus*
Air in the milking unit	at the beginning of milking	–	+/–	–
	in the middle of milking	–	+/–	–
	at the end of milking	+	+/–	+
Flushes from the walls of the milking parlour	at the beginning of milking	–	+/–	–
	in the middle of milking	–	+	–
	at the end of milking	–	+	–
Washes from the skin of cows' udders	before disinfection	+	+	+
	after disinfection	–	+/–	–
Flushing from the rubber of milking cups	at the beginning of milking	–	–	–
	in the middle of milking	+/–	+	+/–
	at the end of milking	+	+	+

The results presented in Table 3 suggest that the growth of *E. coli* and *S. aureus* was not detected in the air of the milking parlour, but in some cases, there was a positive result for the growth of *Salmonella spp.* After the end of the milking period, all identified microorganisms were detected in the air of the milking parlour.

On the other hand, based on the evaluation of samples washed from the walls of the milking hall, it was established that a 0.5% solution of the detergent and disinfectant "Gralan Gel" has a harmful effect on *E. coli* and *S. aureus* and has a weak effect on *Salmonella spp*. This product has a detrimental effect on microbial contamination of the skin of the udder teats of cows for its use in pre-milking disinfection.

Thus, before milking, without performing disinfection, all differentiated sanitary-indicative microorganisms were detected on the skin of the udder of cows. After disinfection, *E. coli* and *S. aureus* were not detected on the skin of the udders of cows, but in some cases *Salmonella spp.* was found. Microbial contamination of the rubber of milking cups had the same dynamics regarding bacterial contamination. Thus, at the beginning of milking, the growth of microorganisms was not observed, but in the middle of the milking period in some samples, and at the end – in all samples, the above-mentioned microorganisms were detected.

Analysis of the results showed that the use of a 0.5% solution of the detergent and disinfectant "Gralan Gel" positively affects the sanitary condition of the milking room, milking equipment, and the skin of the cows' udders.

During the determination of microbial contamination of milking equipment, this study was aimed at comparing the effectiveness of the detergents and disinfectants under study in relation to its various parts (Table 4).

Table 4. General bacterial contamination of milking equipment parts during the use of various detergents and disinfectants, M ± m, n = 5

Product, solution concentration	Washout sampling time	Parts of milking equipment			
		Milking rubber	Collector	Milk hose	Milking machine tank
Before processing milking equipment, thous. CFU/cm^3		371.32 ± 12.4	262.53 ± 10.1	278.48 ± 6.7	231.81 ± 5.7
Soda ash, 0.5%	after treatment. thous.CFU/cm^3	128.15 ± 9.2*	133.56 ± 7.6*	138.62 ± 11.7*	95.35 ± 15.2*
"Desmol", 0.5%	after treatment. thous.CFU/cm^3	58.51 ± 8.2*	25.31 ± 4.7*	47.57 ± 5.9*	35.21 ± 8.1*
"Gralan Gel", 0.5%	after treatment. thous.CFU/cm^3	22.05 ± 2.2*	4.31 ± 1.1*	9.73 ± 2.2*	6.11 ± 0.2*

Note: * P < 0.001, significant compared to pre-treatment indicators for milking equipment

The results presented in Table 4 show that during the application of a 0.5% solution of soda ash, the total bacterial inoculation of the milk gum decreased by 65.5% (P < 0.001), the collector – by 49.1% (P < 0.001), the milk hose – by 5.2% (P < 0.001), the tank of the milking machine – by 58.9% (P < 0.001) compared to the indicators before processing the milking equipment.

At the same time, when using the treatment of dairy equipment with 0.5% "Desmol" solution, the total bacterial contamination of milking rubber decreased by 84.2% (P < 0.001), the collector – by 90.4% (P < 0.001), the milk hose – by 82.9% (P < 0.001), the tank of the milking machine – by 84.8% (P < 0.001) compared to the indicators before the treatment of milking equipment.

Therewith, when using the treatment of dairy equipment with 0.5% "Gralan Gel" solution, the total bacterial contamination of milking rubber decreased by 94.1% (P < 0.001), the collector – by 98.4% (P < 0.001), the

milk hose – by 96.5% (P < 0.001), the tank of the milking machine – by 97.4% (P < 0.001) compared to the indicators before the treatment of milking equipment.

Based on the results of general bacterial contamination of dairy equipment for sanitary treatment, it was also found that the most contaminated was milking rubber (see Table 4). After sanitary treatment of milking rubber with the detergents and disinfectants under study, its total bacterial inoculation decreased by 3.0 times (P < 0.001) – after treatment with a 0.5% solution of soda ash, 6.3 times – after treatment with a 0.5% solution of "Desmol" and 16.8 times – after treatment with 0.5% "Gralan Gel" solution, but it was still the greatest. Obviously, the reason for the high bacterial contamination of milking rubber is that it comes into direct contact with the skin of the udder, which does not always meet veterinary and sanitary requirements, and the environment of the livestock room in which milking is carried out. For a thorough investigation of microbial contamination of the udder skin, it is necessary to use sampling protocols from its various parts and the teat duct. Therewith, varied materials and equipment should be used. This approach allows stating that the microorganisms, which are inside and outside the udders and on the skin of the udder of cattle, differ in their taxonomic composition and number between its different anatomical parts [24].

In previous years, milking tires and milk hoses were replaced four times a year, i.e., once a quarter. Presently, these requirements are not met in farms, which leads to the emergence of cracks that microorganisms can penetrate and multiply. Therewith, according to the study results presented in Table 4, the most effective sanitary treatment of milking equipment occurs when using a 0.5% solution of alkaline detergent and disinfectant "Gralan Gel".

Conclusions

Bactericidal properties of detergents and disinfectants: 0.25% and 0.5% solutions of "Chloramine B", "Sanalcalin", "Neochlor" with active chlorine content – 120 mg/dm^3, 250 and 500 mg/dm^3 have a detrimental effect on the growth of test cultures of *E. coli*, *S. aureus*, and *P. aeruginosa*. 0.5% Desmol solution with an active chlorine content of up to 250 mg/dm^3 had a detrimental effect on the growth of test cultures of *E. coli* and *P. aeruginosa* only 20 minutes in.

The number of microbial colonies in the air of the milking parlour changes during the milking period of cows. Therewith, it is minimal at the beginning and the largest at the end of milking. The dynamics of microbial contamination of the condition of the milking parlour, the skin of milking cows, the udder of cows, and the rubber of milking cups without the use of detergents and disinfectants leads to the detection of *E. coli*, *Salmonella spp.*, and *S. aureus*.

The use of a 0.5% solution of the alkaline detergent and disinfectant "Gralan Gel" resulted in a detrimental effect on *E. coli* and *S. aureus* and a minor effect on *Salmonella spp.* This product has a detrimental effect on microbial contamination of the skin of the udder teats of cows for its use in pre-milking disinfection.

Thus, treatment of milking equipment with a 0.5% "Gralan Gel" solution leads to a reduction of the total bacterial inoculation of the milking rubber by 94.1%, the collector – by 98.4%, the milk hose – by 96.5%, the tank of the milking machine – by 97.4% compared to the indicators before the treatment of milking equipment with detergents and disinfectants.

Future studies aim to investigate the use of various concentrations (1-10%) of the alkaline detergent and disinfectant "Gralan Gel" with different degrees of contamination of the udder of cows.

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Ефективність застосування мийно-дезінфікуючих засобів під час виробництва молока корів

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Анотація. Актуальність дослідження зумовлена необхідністю покращення гігієни доїння корів, шляхом підбору мийно-дезінфікуючих засобів із різним компонентним складом, що забезпечує якість і безпечність молочних продуктів. Мета цієї роботи полягала у дослідженні ефективності дії розчинів сучасних мийно-дезінфікуючих засобів, що містять в своєму складі активний хлор і безхлорних, на тест-культури мікроорганізмів, санітарно-гігієнічний стан доїльної зали, частини доїльного обладнання та шкіру дійок вим'я корів. Визначали загальну кількість бактерій за вимогами ДСТУ ISO 15214:2007, шляхом посів у 1 см^3 дослідного матеріалу на м›ясо-пептонний агар з наступною інкубацією за середньої температури 36 ºС упродовж 24-48 год. Культивування проводили в термостаті за температури 37 ºС. Після інкубації здійснювали підрахунок колоній мікроорганізмів, що виросли, та визначали кількість колонієутворюючих одиниць. Мийно-дезінфікуючі засоби «Хлорамін Б», «Sanalcalin» і «Неохлор» згубно діють на ріст тест-культур *E. coli*, *S. aureus* і *P. aeruginosa*. «Дезмол» пригнічує ріст тест-культур *E. coli* і *P. aeruginosa* через 20 хв. Кількість колоній мікроорганізмів в повітрі доїльної зали мінімальна на початку та найбільша – в

кінці доїння. 0,5 % розчин згубно діє на *E. coli* та *S. aureus* і незначно впливає на ріст *Salmonella spp*. Водночас цей засіб виявляє ефективність щодо мікробного забруднення шкіри дійок вимені корів за його використання у переддоїльній дезінфекції. Під час обробки доїльного обладнання 0,5 % розчином «Gralan Gel» загальне бактеріальне обсіменіння дійкової гуми зменшується на 94,1 %, колектору – на 98,4 %, молочного шлангу – на 96,5 %, бачку доїльного апарату – на 97,4 % порівняно з показниками до обробки доїльного обладнання мийно-дезінфікуючими засобами. За результатами дослідження найкращу ефективність щодо зниження загального бактеріального забруднення доїльного обладнання, згубної дії на умовно-патогенну мікрофлору і мікробне забруднення шкіри дійок вимені корів за використання у переддоїльній дезінфекції на підприємствах з виробництва молока виявляє 0,5 % розчин лужного мийно-дезінфікуючого засобу «Gralan Gel». Таким чином, 0,5 % розчин лужного мийно-дезінфікуючого засобу «Gralan Gel» доцільно використовувати виробникам молока-сировини коров'ячого для зменшення загального бактеріального обсіменіння доїльного обладнання та вим'я корів

Ключові слова: промислове скотарство, дезінфекція, бактерицидні властивості, мікробне забруднення

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Immunosuppressive Activity of *Campylobacter Jejuni* Isolates in Relation to the Cellular Link of the Body's Immunoprotection

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Abstract. Global environmental changes have caused transformations in the biology of microorganisms, especially among campylobacter, which are currently associated with food toxic infections. The means of influence of these bacteria on susceptible organisms, namely toxins, have not been finally clarified. The purpose of this study was to investigate the genetic conditionality of toxin formation in isolates of *Campylobacter jejuni* and determination of the degree of inhibition of the body's protective reactions by toxic fractions of Campylobacter protein compounds. The methodology of this study was based on the polymerase chain reaction using primers to indicate the nucleotide sequences of the *Campylobacter jejuni* genome that encode the synthesis of toxins. Samples from 4 Campylobacter isolates were examined for the content of protein fractions according to the Lowry assay. The analysis of the electropherogram of the results of DNA amplification in a comparative aspect with the data of standard samples allowed establishing the presence of genome elements that indicate the potential ability to produce toxins in *Campylobacter jejuni* isolates sampled from the material under study. Toxic fractions separated from the supernatant of *Campylobacter jejuni* broth culture are represented by protein-carbohydrate substances. The obtained peak toxigenic fractions of the dialysate of the bacterial culture sediment contained protein within 9.5-17 μg/ml. In the dialysate of the broth culture supernatant, where 5 groups of toxigenic fractions were distinguished, their protein content ranged within 10-85 μg/ml. By reproducing the opsono-phagocytic reaction involving toxigenic fractions of *Campylobacter jejuni*, a sufficiently pronounced immunosuppressive effect of these complexes on the body of warm-blooded animals was established with an opsonic index of 2.6 ± 0.03. The obtained results allow clarifying the connection between toxin formation in *Campylobacter jejuni* and their immunosuppressive effect on the body of warm-blooded animals and humans, which in the future will positively affect the improvement of measures for the prevention and treatment of animals with this pathology

Keywords: campylobacter isolates, DNA amplification, protein fraction, opsono-phagocytic reaction

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Introduction

Campylobacteriosis is the most common foodborne bacterial zoonosis in the world, caused by *Campylobacter fetus, jejuni*, and *coli.* EU countries carry out annual monitoring of the spread of campylobacter among animals and poultry. This infectious pathology is caused by *Campylobacter fetus* and *jejuni.* During infection, campylobacter act in many ways, namely through toxins. As a food toxic infection, campylobacteriosis is registered in many regions of the world [1; 2].

The national medical associations of the countries of the world, as well as the World Health Organization (WHO), emphasize the special role of *Campylobacter* in the aetiology of food toxic infections and attribute them to a single series of classical enteropathogenic genera of bacteria, such as *Salmonella, Shigella*, and *Yersinia* [3; 4]. This is caused by the extraordinary geographical distribution of *Campylobacter*, high rates of morbidity and intensive circulation of the pathogen among animals and certain social groups of the population [5; 6]. Separate studies indicate a high degree of carriage of campylobacteriosis in cattle, pigs, dogs, and poultry against the background of a significant spread of campylobacterial diarrhoea in humans [6; 7]. The most pathogenic are Campylobacter of the *fetus* and *jejuni* subspecies, as well as the *coli* species [8; 9].

Campylobacter is included in group No. 2, which combines aerobic (microaerophilic, motile, spiral) and gram-negative bacteria [10]. The genus *Campylobacter* includes 15 species of bacteria: *fetus, hyointestinales, jejuni, cinaedi, coli, concisus, cryaerophila, fennelie, lari, mucosalis, nitrofigillis, sputorum, upsaliens*, as well as *Weilonella curva* and *Weilonella succinogenes* species added to this genus. In turn, some species combine subspecies and biovars. Thus, the *fetus* species unites the *fetus* and *venerealis* subspecies, the *jejuni* species – *jejuni* and *doylei* subspecies, the *sputorum* species – *sputorum, bubulus*, and *fecalis* biovars [11].

Campylobacter bacteria are divided into catalase-positive (*C. fetus* with subspecies: *spp. venerialis, spp. fetus; C. Jejuni* with subspecies: *spp. jejuni, spp. doyli; C. coli; C. lari; C. hyointestinalis*) and catalase-negative (*C. sputorum* with two biovars: *sputorum* and *bubulus*, *C. mucosalis*) [5]. Some species that previously belonged to the genus *Campylobacter* are now separated into a new genus. Thus, *C. piloridis*, associated with gastric ulcer disease, is separated into an independent genus of *Helicobacter*. The range of microbial species that are candidates for inclusion in this taxon is also constantly increasing [10; 12]. The antigenic composition of *Campylobacter* is quite complex and contains H-, O-, and K-antigens [13].

Important surface antigens are lipopolysaccharide and acid-soluble protein fraction. These antigens play a leading role during serotyping of *C. hepaticus* and *C. jejuni* and serodiagnosis of campylobacteriosis. The question of the existence of a "universal" *Campylobacter* antigen, i.e., such an antigen, the antibodies to which can be detected in the serum of all patients in sufficiently high titres, stays open. But all *Campylobacter* serotypes have a common protein antigen with a molecular weight of about 62 kDa. This protein fraction is optimal for identification of campylobacteriosis during serological diagnosis [14].

Antigenic differences between bacteria of different serotypes relate to the carbohydrate composition of the internal lipopolysaccharide of *Campylobacter* [15]. The chemical composition of lipopolysaccharide of *Campylobacter jejuni* is similar to analogous antigens of other enterobacteria. Apart from the similarity of the chemical structure, the existence of a significant immunological affinity between lipopolysaccharides of *Campylobacter* and other pathogens of intestinal infections – *Salmonella, Yersinia, Brucella, Shigella* [16] has been proven.

Campylobacter is endowed with a powerful list of virulence factors that cause intoxication of the body and predetermine the development of pathological processes with distinct clinical manifestations [17]. The issue of *Campylobacter* toxin production is of important practical and theoretical importance [18].

Like all Gram-negative bacteria, *Campylobacter* contains endotoxins. It is known that the endotoxin of Gram-negative bacteria is a lipopolysaccharide of the cell wall [15]. Even though, in experiments, the thermostable endotoxin of *Campylobacter* can cause haemorrhagic and necrotic changes in the skin at the places of introduction of the bacterial suspension and even the death of animals, its role in the pathogenesis of campylobacter enteritis is not considered leading [19].

The ability of various species of *Campylobacter* to produce a thermolabile enterotoxin, which has antigenic affinity with cholerogen and enterotoxin of *E. coli*, is generally recognized [18]. Bacterial toxins can cause an inflammatory reaction of the skin, spleen hyperaemia, necrosis of lymphoid elements, and in blood vessels – thrombosis and intravascular coagulation, collapse, and haemoglobin enrichment of their walls [20].

In addition to endotoxins and enterotoxins, *Campylobacter* synthesizes cytotoxins. A considerable number of such compounds were found among highly cytopathogenic "chicken" strains (44.1%), which determined the leading role of chickens as a potential source of pathogens of campylobacteriosis [7; 21].

The susceptibility of the macroorganism to campylobacteriosis causes immunological disorders, including various immunodeficiency states [22]. Although the study of the issue of toxin formation among microorganisms is quite popular, the specific features of toxin formation in Campylobacter depending on the virulence of the pathogen, the nature of the manifestation of the toxic effect of these objects *in vivo* are still unclear, which forms the relevance of this area of research.

The purpose of this study was to identify the degree of inhibition of the body's defence reactions by toxic fractions of Campylobacter protein compounds and to investigate the genetic conditionality of toxin formation in *Campylobacter jejuni* isolates.

Materials and Methods

The study was conducted at the State Research Institute of Laboratory Diagnostics and Veterinary-Sanitary Examination (Kyiv) during 2021-2022 per ISO 10272-1:2017 (Microbiology of the food chain – Horizontal method of detection and counting of *Campylobacter spp*. – Part 1: Detection method) [23].

From 2,120 samples of faeces and droppings, caecum (caecal processes) with contents from cattle, pigs,

and poultry, obtained from farms of various forms of ownership in Ukraine during 2021, 448 isolates – pathogens of zoonoses and commensal microorganisms – were isolated and identified, which is 21.1% of the total number of samples. 33 isolates of *Campylobacter jejuni* were typed according to general biological properties. According to the source of isolation: from poultry – 26, cattle – 5, pigs – 2, from which 4 isolates of *Campylobacter jejuni* were selected for work.

During the study, certified nutrient media and selective additives (horse blood serum) were used. Antibiotic susceptibility of isolates was investigated using the disk diffusion method, according to EUCAST recommendations [24].

The sample preparation sequence for obtaining DNA from campylobacter field isolates included several steps. 100 µl of a 48-hour broth culture of bacteria washed with buffered physiological solution (BPS) was placed in microcentrifuge tubes (Eppendorf type) with a capacity of 1.5 cm^3. Then 300 µl of lysis buffer containing 6M guanidine thiocyanate was added to each sample and mixed with a vortex. The test tubes with the mixture were heated at 65°C for 5 minutes and re-mixed using a vortex. The sample material was precipitated by centrifugation at 5 x 10^3 rpm for 5 seconds. Subsequently, 30 µl of silica gel was added to each sample. The mixture was vortexed for 10-15 seconds and left at rest at room temperature for 2 min until the sorbent turned into sediment. The samples were then re-mixed and settled for 5 minutes.

To separate the sorbent fractions with the material under study and solvents, the test tubes were centrifuged at 5,000 rpm for 30 seconds. Each sample was washed first with 300 µl of lysis solution, and then twice with 500 µl. The mixture consisted of 70% ethanol, 100 mM NaCl, and 10 mM Tris-HCl (pH 8.0). The supernatant was removed. The precipitated substance was dried for 5-7 minutes under a thermostat at 65°C. Subsequently, 50 µl of TE buffer (pH 8.0) was added to each Eppendorf for DNA elution and placed in a thermostat at 65°C for 5-6 min. During elution, the samples were vortexed every minute. The substance was again precipitated by centrifugation at 12,000 rpm for 2 minutes. The supernatant containing the DNA obtained for further research was collected in pre-prepared test tubes.

Before setting up the polymerase chain reaction (PCR), two reaction mixtures were prepared – "upper" and "lower". The "lower" reaction mixture contained 2.5 µl of primer and nucleotides, mixed on a vortex, with a final concentration of each primer of 25 rMol/sample. The primers used in the study were manufactured by Wizard® (USA). 5 µl of each obtained mixture of the corresponding sample was placed in 0.5 cm^3 microtubes, followed by the application of 10 µl of wax melted at 95°C to the surface until the entire surface was completely covered. The composition of the "upper" reaction mixture included, considering controls, 10.0 µl of 5% PCR buffer, 3.0 µl of 50 mM $MgSO_4$, 6.0 µl of H_2O, 1.0 µl of Taq polymerase. In test tubes with the "lower" reaction mixture, the surface of the wax layer was covered with 10 µl of the "upper" reaction mixture and, according to the sample labelling, 10 µl of experimental or control DNA and two drops of petroleum jelly were applied. Mixtures of DNA of somatic cells of agricultural animals (cattle, pigs, sheep) and DNA of *Escherichia coli* and *Salmonella typhimurium* were used as negative control during amplification. The polymerase chain reaction was performed according to the program using the thermal cycler "Tertsik" in the active control mode.

Determination of the nature of the toxic component of *Campylobacter jejuni* isolates was based on the establishment of its biochemical nature. To obtain the exotoxin, the protein fractions of the culture liquid of Campylobacter, grown for 48 h at 42°C in Preston's broth, were extracted. After precipitation of bacterial cells by centrifugation at 3,000 rpm for 30 minutes, the supernatant obtained from the samples was transferred to clean test tubes. Protein fractions of each sample were isolated by adding up to 60% saturation of dry ammonium sulphate salt to each of the test tubes with simultaneous pH control. Denatured proteins of the samples were obtained as a result of settling the mixtures for 12 h at 4°C, centrifuged at 3,000 rpm for 30 min and dissolved in 3 M ammonium sulphate in a ratio of 1:3.

The quantitative content of the isolated proteins was investigated using the Lowry protein assay [25]. Samples from each of the 4 isolates of Campylobacter included 5 samples: culture fluid, supernatant after protein precipitation of culture fluid (4.14 mg/ml), supernatant dialysate (1.015 mg/ml), sediment (0.397 mg/ml), and sediment dialysate (0.23 mg/ml). The constituents of the sediment and supernatant were detected using ion exchange chromatography on TSK gels.

Ion exchange chromatography of the sample (e.g., sediment) was performed on a column (2 × 35 cm) Fractogel DEAE-650-s "Merck" (Germany), equilibrated with 0.01 M Tris-HCl buffer (pH 7.0). The sample (20 ml, 50 mg of protein) was applied to the column, eluted with a linear gradient of NaCl (0-1 M, 150 ml each) at 24 ml/h. Substances identical to the fractions of the obtained peaks were tested for toxic activity. The harmful effect of *Campylobacter jejuni* toxin on the body was studied by determining the opsonic index.

Under sterile conditions, a mixture of 0.1 ml of rabbit blood and 0.1 ml of 2% sodium citrate solution was introduced into centrifuge tubes. The test tubes were labelled according to the campylobacter isolate under study, and 0.1 ml of 2 billion suspensions of the washed agar culture of *Campylobacter jejuni* bacteria were added to the citrated rabbit blood in a certain test tube. In another test tube with similar components, 0.1 ml of a certain fraction of toxin-containing substances obtained from the supernatant of *Campylobacter jejuni* broth culture was added. The reagent tubes were kept for 30 minutes in a thermostat at 37°C, then centrifuged at 3,000 rpm and the supernatant with white blood cells was selected with a pipette. Smears were prepared from the leukocyte-containing substance and stained according to the Romanowsky-Giemsa method [4] to count phagocytic bacteria in toxin-containing and toxin-free fractions.

Ion exchange chromatography of the sample (supernatant) was performed using a column (3.0 × 40 cm) Fractogel DEAE-650-m "Merck" (Germany), equilibrated with 0.01 M Tris-HCl buffer (pH 7.0). The sample (10 ml, 40 mg of protein) was applied to the column and eluted using a linear NaCl gradient (0-1 M, 150 ml each) at 30 ml/h. Substances identical to the fractions of the obtained peaks were tested for toxic activity. Statistical analysis was performed according to the program R: A Language [26].

Results and Discussion

All 33 typed field isolates of Campylobacter compared to the reference strain *Campylobacter jejuni* ATCC 33291 demonstrated similar biochemical properties – they produced oxidase, catalase, and urease, decomposed hippurate, and showed growth at +37°C and +42°C (Table 1). This is consistent with the results of studies by E. Lucio, I. Sakaridis, and L. García-Sánchez [5; 6; 10].

Table 1. Differential features of pathogenic isolates of *Campylobacter jejuni*

Isolate	Origin	Oxidase	Catalase	Urease	Growth at +25°C	Growth at +37°C	Growth at +42°C	Hippurate Na	Cephalothin
C. jejuni No. 1	Broiler chicken	+	+	–	–	+	+	+	sensitive
C. jejuni No. 2	Broiler chicken	+	+	–	–	+	+	+	sensitive
C. jejuni No. 3	Broiler chicken	+	+	–	–	+	+	+	sensitive
C. jejuni No. 4	Broiler chicken	+	+	–	–	+	+	+	sensitive
C. jejuni ATCC 33291	Reference strain	+	+	–	–	+	+	+	sensitive

Careful verification of the basic cultural, morphological, and biochemical properties of field isolates is extremely necessary since these data are essential when working with experimental material. They confirm the work with a pure bacterial culture.

Under unfavourable conditions, *Campylobacter* species are transformed from the classic mobile spiral form into a ball-shaped one. Therewith, they stay workable, but transition into an uncultivated state. The use of media with elements of animal blood during the study helped speed up the adaptation time of the isolates to artificial cultivation conditions. In the presence of organic iron compounds in the nutrient medium, the cell membranes of the bacterial wall become more resistant to the previously mentioned stresses.

As the experience of many scientists proves [1], the use of PCR testing in the search for agents and products of their metabolism is more effective compared to cultural methods, especially if it concerns *Campylobacter spp.* To identify the toxin-synthesizing ability in a comparative aspect, the order of the DNA nucleotide sequences of each of the 4 selected isolates of *Campylobacter jejuni* was analysed. To identify specific oligonucleotide primers, data on DNA sequences of campylobacter were used according to the MLST principle. They are registered in the international GenBank database and implemented using Pub MLST [27] and Genome Profiler software. The search resulted in primers with the following nucleotide sequences: F (ATGAAAAAATATTTAGTTTTTGCA) and R (ATTTTATTATTTGTAGCAGCG) (Table 2).

Table 2. Temperature regimes of the amplifier for PCR reproduction using forward and reverse primers

Primers F: ATGAAAAATATTTAGTTTTTGCA R: ATTTTATTATTTGTAGCAGCG		
Stage	**Mode**	**Number of cycles**
1	t 95°C – 5 min	1
2	t 94°C – 1 min t 56°C – 1 min t 72°C – 2 min	– 4 –
3	t 94°C – 30 s t 55°C – 30 s t 72°C – 30 s	– 28 –
4	t 75°C – 5 min	1
5	t 10°C	1

Such a nucleotide sequence corresponds to one of the genes (cdtA), as a result of which the synthesis of the so-called cytolethal stretching toxin takes place [22]. This gene is included in the triad of genes (cdtA, cdtB, and cdtC) necessary for the synthesis of an active ternary holotoxin, which causes full cellular toxicity.

Genomic features of field isolates using the primer responsible for the synthesis of the toxic protein fraction of *Campylobacter jejuni* were examined for the presence of potential genes in each isolate and the reference strain [14].

The specific feature of the PCR reproduction in this case concerned the installation of Eppendorfs and the program launch, starting with recording of the "hot star" conditions in the thermal cycler (temperature +93°C). The study and analysis of compounds obtained as a result of amplification began after separating a section of DNA in 1.5% agarose gel. An indispensable condition for using agar gel is its enrichment with a solution of ethidium bromide at 25 μL per plate with a thickness of 6 mm.

At the stage when the reaction mixture for PCR already contained glycerol, and for marker staining it contained xylenecyanol, the samples under study were introduced directly into the wells. In the electrophoretic chamber, the gel strips were placed in wells towards the anode. 10.0 μl of amplified samples were placed in the central wells, and 3 μl of marker was placed in the extreme wells. After the dye (xylenecyanol) covered about half the length of the gel, electrophoresis was performed in a voltage gradient of 10 V/cm. The calculation of the results of DNA amplification on electropherograms using a transilluminator in ultraviolet light is presented in Figure 1.

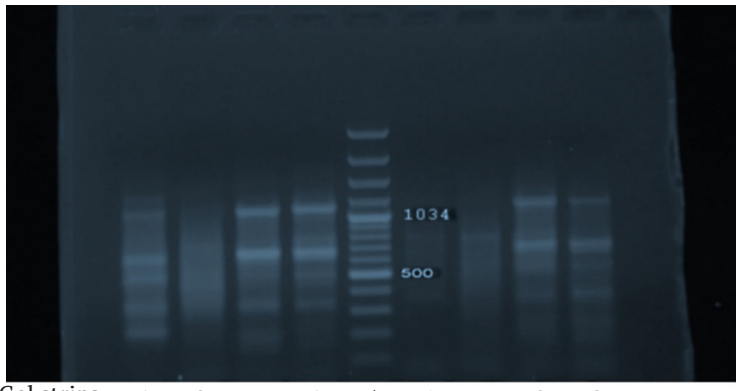

Figure 1. Results of electrophoresis of amplification of toxicity genes in the DNA of *Campylobacter jejuni* isolates

Comparative analysis of the electropherogram of DNA amplification with the data of standard samples as a result of three repetitions helped establish the presence of genome elements ($P < 0.01$) that indicate the potential ability to produce toxins in *Campylobacter jejuni* isolates sampled from the material under study (lanes 1 to 4). The fifth lane coincided with the sample of the reference strain *Campylobacter jejuni* ATCC 33291. Empty tracks 6, 7, 8, and 9 belong to samples containing control samples (without the presence of Campylobacter and somatic cell cultures in poultry).

Therefore, the gene of interest (cdtA) was identified as specific for *Campylobacter jejuni*. It was present in all 100% of isolates (four isolates) and was not detected in the control negative samples. These are samples with epithelial cells and samples containing cultures of *Escherichia coli* and *Salmonella typhimurium*, which is quite natural.

Since, according to statistics, among the three most common intestinal infections associated with the presence of toxin-producing bacteria (*Shigella sonnei, Campylobacter spp., Salmonella spp.*), Campylobacter is ahead of salmonella; some scientists point to the need to investigate toxin production by Campylobacter [4]. Campylobacter came under the close attention of specialists because they are included in the top-5 group of pathogens of intestinal zoonoses, which can spread through animal faeces [5].

The study of the nature of the toxic products of the sediment and the supernatant of the broth culture of selected Campylobacter strains is presented in Figure 2 and 3, respectively.

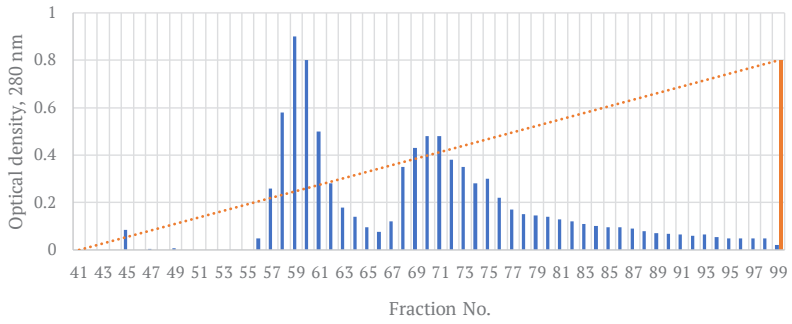

Figure 2. Distribution of protein fractions of the precipitate of *Campylobacter jejuni* isolate No. 1 (ion exchange chromatography)

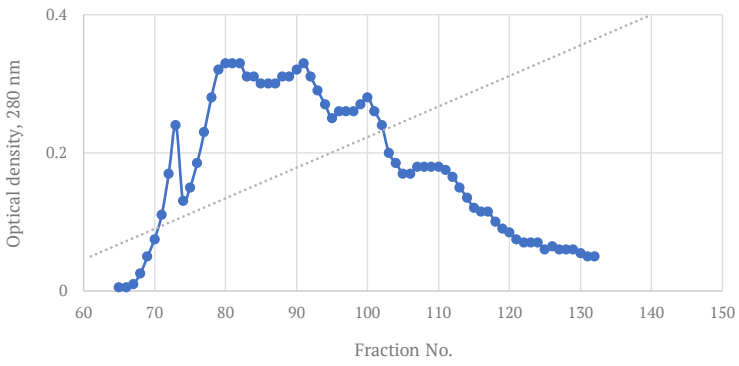

Figure 3. Distribution of protein fractions of the supernatant of *Campylobacter jejuni* isolate No. 1 (ion exchange chromatography)

The two peak values of the protein components of the sediment coincide with the 59th and 70th fractions.

The results of studies of the supernatant of a *Campylobacter jejuni* isolate with potential toxic properties allowed identifying 5 protein fractions, which constitute a considerable advantage over other substances of this class. These are fractions No. 73, 80, 91, 100, and 105. Data on the quantitative and qualitative biochemical composition of samples of elements of the supernatant of Campylobacter isolates, which were subject to verification, are presented in Table 3.

Table 3. Protein content in sediment and supernatant fractions of *Campylobacter jejuni* No. 1 (M ± m, n = 6, P < 0.01)

Sample	Protein content, μg/ml
Sediment (dialysed)	238
Fraction 59	17
Fraction 70	9.5
Supernatant (dialysed)	242.5
Fraction 73	24.5
Fraction 80	85
Fraction 91	55
Fraction 100	20
Fraction 105	10

The secretion of a protein substance by Campylobacter is activated due to the contact of the bacteria with the host cells. These are the so-called Cia proteins, which are necessary for activation of chemotaxis and mass invasion of intestinal epithelial cells by *Campylobacter jejuni* bacteria. This is consistent with literature data on the system that implements the synthesis of Cia proteins and is in the flagellar apparatus of these microorganisms. It is associated with the activation of the diguanylate cyclase enzyme [15]. Intensification of the activity of the latter helps alter the biological state of the Campylobacter culture. There is a transition from the swarming phase to the phase with the development of morphologically stem-like cells. This phase involves the removal of flagella from bacterial cells and symbolizes the beginning of cell division [13].

Apart from enzyme proteins, a crucial factor in the pathogenic effect on the body in *Campylobacter jejuni* is cytolethal distension toxin (CdtA, CdtB, CdtC). It causes apoptosis of host cells, which is accompanied by a similar phenomenon-elongation of affected cells [22]. The use of isolated toxic fractions of protein compounds of *Campylobacter jejuni* isolates in the suspension of the leukocyte fraction of rabbit blood led to the occurrence of a similar effect – apoptosis with cell elongation. This can explain the principle of the immunosuppressive effect of cytoletal stretching toxin on the activity of white blood cells that lose their phagocytic function.

The degree of such changes can be estimated by the result of an opsonophagocytic reaction. The number of bacterial cells captured by phagocytes (phagocytic number) for n = 6 in the sample with sediment of *Campylobacter jejuni* bacterial culture (isolate No. 1) was 530 ± 12 microbial cells per 100 leukocytes, which is equal to 5.3 ± 0.12.

In the presence of the supernatant of the *Campylobacter jejuni* bacterial culture (isolate No. 1), the phagocytic number in the sample for n = 6 did not exceed 202 ± 4 microbial cells per 100 leukocytes, which is equal to 2.0 ± 0.04.

The analysis of the obtained data indicates that outside the body, rabbit blood leukocytes treated with the protein fraction of the dialysed sediment of *Campylobacter jejuni* No. 1 with a protein content of 238 μg/ml showed little functional activity. Their ability to absorb *Campylobacter jejuni* bacterial cells by phagocytosis was over 2.6 times less than that of leukocytes treated with the supernatant of the same isolate with a protein content of 242.5 μg/ml. This indicates a high cytotoxic effect of protein compounds of the dialysed supernatant, which consists of 5 fractions.

Such a difference in the immunosuppressive action of protein fractions of *Campylobacter jejuni* bacterial cultures of different origins was confirmed by the calculation of the opsonic index (I_o) based on the quantitative indicators of phagocytic numbers of the 1st and 2nd groups of the experiment. It was as follows: $I_o = (5.3 \pm 0.12)/(2.0 \pm 0.04) = 2.6 \pm 0.03$.

Thus, the obtained quantitative characteristics of the interaction of various components of the bacterial culture of *Campylobacter jejuni* with a suspension of rabbit blood leukocytes indicate the manifestation of the cytotoxic effect of the Campylobacter supernatant, which contains toxigenic protein fractions. Since such an effect is observed when interacting with immunocompetent cells, the toxic compounds obtained in the experiment can be qualified as factors with an immunosuppressive cellular effect. The obtained results are fully consistent with the data of other scientists [14; 19], which confirm the acquisition of plasmids in the genome by Campylobacter, which cause the synthesis of protein compounds with pronounced cytotoxic properties.

Conclusions

As a result of the study of 33 isolates of *Campylobacter jejuni* from poultry (faeces and caecal processes of the intestine), 4 cultures were selected to establish the presence of the cdtA gene. Only these cultures were similar in biochemical properties to the reference strain *Campylobacter jejuni* ATCC 33291.

A positive result was obtained for the PCR study to detect the cdtA gene in 4 isolates of *Campylobacter jejuni* in a comparative aspect, along with samples of somatic cells and cultures of *Escherichia coli* and *Salmonella typhimurium*. The presence of this gene confirms the presence of toxin-forming ability in Campylobacter.

The effect of *Campylobacter jejuni* waste products on the body of warm-blooded animals was studied by detecting changes in the phagocytic activity of rabbit blood leukocytes due to their susceptibility to the protein fraction of the dialysed supernatant and the dialysed campylobacter sediment (isolate No. 1). The cytotoxic activity of the dialysed supernatant with a protein content of 242.5 μg/ml exceeded the intensity of inhibition of the phagocytic function of blood cells treated with the dialysed sediment of the same broth culture with its content of 238 μg/ml.

The calculated level of potential blocking of the opsono-phagocytic reaction by *Campylobacter jejuni* culture exchange products corresponded to the opsonic index value of 2.6 ± 0.03. For the successful treatment and prevention of infections caused by *Campylobacter jejuni*, it is necessary to constantly monitor the presence of toxin-producing strains in sensitive organisms.

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Імуносупресивна активність ізолятів *Campylobacter jejuni* щодо клітинної ланки імунопротекції організму

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Анотація. Глобальні зміни довкілля викликали трансформації в біології мікроорганізмів, зокрема й серед кампілобактерій, котрі асоціюються нині з харчовими токсикоінфекціями. Інструменти впливу цих бактерій на сприйнятливі організми, зокрема токсини, остаточно не з'ясовані. Метою дослідження стало вивчення генетичної обумовленості токсиноутворення в ізолятів *Campylobacter jejuni* та визначення ступеня пригнічення захисних реакцій організму токсичними фракціями білкових сполук кампілобактерій. Основою методології досліджень була полімеразна ланцюгова реакція з використанням праймерів для індикації нуклеотидних послідовностей геному *Campylobacter jejuni*, що кодують синтез токсинів. Зразки проб від 4-х ізолятів кампілобактеру досліджували на вміст білкових фракцій за методом Лоурі. Аналіз електрофореграми результатів ампліфікування ДНК в порівняльному аспекті з даними стандартних зразків дозволив встановити наявність елементів геному, які вказують на потенційну здатність до токсиноутворення у виділених з досліджуваного матеріалу ізолятів *Campylobacter jejuni*. Токсичні фракції, відокремлені з супернатанту бульйонної культури *Campylobacter jejuni*, представлені речовинами протеїново-вуглеводної природи. Отримані пікові токсигенні фракції діалізату осаду бактерійної культури містили в собі білок у межах 9,5–17 мкг/мл. У діалізаті супернатанту бульйонної культури, де було визначено 5 груп токсигенних фракцій, уміст в них білка коливався в межах від 10 до 85 мкг/мл. За відтворення опсоно-фагоцитарної реакції із залученням токсигенних фракцій *Campylobacter jejuni* встановлено достатньо виражену імуносупресивну дію цих комплексів на організм теплокровних тварин при опсонічному індексі 2,6 ± 0,03. Отримані результати дають змогу з'ясувати зв›язок між токсиноутворенням у *Campylobacter jejuni* та їх імуносупресивною дією на організм теплокровних тварин і людини, що в перспективі позитивно впливатиме на удосконалення заходів щодо профілактики та лікування тварин за цієї патології

Ключові слова: ізоляти кампілобактерій, ампліфікація ДНК, білкова фракція, опсоно-фагоцитарна реакція

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Morphological and Biochemical Changes in Blood Parameters in Cats with Infectious Peritonitis

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Abstract. Feline infectious peritonitis is a feline disease, the causative agent of which is a mutant coronavirus, which leads to the death of young animals. Presently, this pathology is considered incurable, and therefore it requires a detailed study. The purpose of this study was to establish the features of clinical manifestation and haematological parameters in cats with infectious peritonitis. This paper presents the results of a clinical and laboratory study of 12 cats aged from 6 months to 3 years who were diagnosed with effusive and dry infectious peritonitis. Clinical, laboratory, and visual research methods were used. Laboratory analysis included a study of morphological and biochemical parameters of blood, cytological examination of effusion from the abdominal and pleural cavities, and a Rivalta test. As a result of cytological examination of effusion from these cavities, a high concentration of cells, macrophage accumulation, neutrophil phagocytosis, and red blood cells were detected in the entire field of view. It was found that the predictive value of the Rivalta test for feline infectious peritonitis is 62.5%. According to haematological examination, all sick animals were diagnosed with anaemia, lymphopenia, hypoalbuminemia, hyperglobulinemia, hyperbilirubinemia, increased activity of relative liver-specific enzymes (aspartate aminotransferase, alkaline phosphatase). Trobmocytopenia was observed in two animals, and an increase in blood markers of the functional state of the kidneys (creatinine, urea) was found in one cat. The serum albumin/globulin ratio should also be considered an important diagnostic indicator. Thus, with feline infectious peritonitis, this indicator should be < 0.4. In 11 sick cats, this ratio was characterized by values below 0.4, and in one animal this indicator was 0.46. In general, the obtained data of morphological and biochemical blood tests are not specific for feline infectious peritonitis, and therefore the authors of this paper recommend their comprehensive investigation for diagnostic purposes. An objective assessment of the available methods of laboratory diagnostics will contribute to the creation of a diagnostic protocol for feline infectious peritonitis

Keywords: coronavirus infection, clinical and haematological studies, Rivalta test, cytological examination

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Introduction

Coronaviruses cause respiratory and gastrointestinal diseases in most animal species. In cats, the coronavirus (FCoV) is represented by two biotypes, commonly referred to as feline intestinal coronavirus (FeCV) and feline infectious peritonitis virus (FiPV). It is believed that FeCV is associated with the development of a mild form of gastrointestinal infection, but cases of mild respiratory infections have also been established. It is known that the initial infection with the FCoV occurs by faecal-oral route. In some cats infected with FCoV, a mutation in the viral genome leads to a change in the tropism of the FCoV host cell from enterocytes to macrophages, which provokes a systemic infection known as feline infectious peritonitis (FIP) [1].

Feline infectious peritonitis is a disease with a high mortality rate that occurs in domestic and wild members of the feline family all over the world. The aetiological agent of the disease, feline coronavirus (FCoV), occurs in two different pathogenic types, which can be distinguished by their biological behaviour, but not by their morphology [2]. Presently, FIP stays a disease of cats with a 100% mortality rate and is understudied in veterinary practice [3].

This infectious pathology of cats is still one of the least researched viral diseases. Thus, for fifty years, it turns out to be a disease for which there is no diagnostic test, no vaccine for prevention, and no clear understanding of the mechanism of interaction of the virus with the body of a sensitive animal during the occurrence of the disease [4].

FIP can affect animals regardless of age but is most common in cats younger than 3 years and especially between 4 and 16 weeks of age. The disease is most often diagnosed in kennels (breeding cats), animal shelters, and places with a high concentration of animals per 1 m^2. Typically for an enzootic infection, the incidence of feline infectious peritonitis can vary widely over time. Mortality is very high, especially after the onset of clinical symptoms, although some cats can live with this disease for several weeks, months, or, in rare cases, years [11; 12].

Diagnosis of feline infectious peritonitis is quite complicated. This is explained by the fact that FIP does not have specific clinical symptoms. However, N. Pedersen [4; 5] states that the diagnosis of FIP is based primarily on consideration of the age of the animal, its origin, keeping conditions, the presence of other patients in contact, clinical symptoms, and physical examination data by a veterinary specialist. Cats aged 4-36 months who are surrounded by many other animals that show a persistent but undulating fever that does not respond to the use of distinct types of antibiotics should, first of all, be suspected of having FIP. More specific symptoms of FIP, which are noticed by owners or detected during a physical examination, will further narrow the range of diseases. Distension of the abdominal cavity due to the accumulation of exudate, dyspnoea in the presence of exudate in the pleural cavity, icterus of visible mucous membranes and skin, hyperbilirubinemia, visible masses on the kidneys and/or mesenteric lymph nodes, uveitis, and some other neurological symptoms associated with involvement of the brain or spinal cord in case of dry form of FIP. In 2019, N. Pedersen *et al*. [5] found that there is a group of preparations for FIP treatment.

The purpose of this study was to find the most characteristic changes in the morphological and biochemical indicators of blood and establish the features of the cytological pattern of exudate from the pleural and abdominal cavities in cats diagnosed with FIP.

Literature Review

S. Tasker [6] found that natural FCoV infections are transient in about 70% of cats, while persistent infection occurs in only 13% of animals. It is the latter that are permanent carriers of the virus. In most cases, coronavirus infection in cats is asymptomatic or manifests itself in minor disorders in the functioning of the gastrointestinal tract (for instance, diarrhoea, vomiting, and anorexia). Although occasionally, there is a severe course of the disease with damage to the gastrointestinal tract. It is also known that about 5-10% of cats are resistant to FCoV.

L. Golovko *et al*. [7] established the genetic component of GWAS and the predisposition of Burmese cats to FIP. F. Riemer [8] indicated epizootological features of feline infectious peritonitis. According to Riemer, FIP was more common in young males. Therewith, the status of castration was not associated with the development of the disease. Furthermore, no breed predisposition to FIP was found. Most of the cats belonged to the breed of domestic short-haired and mongrel cats.

N. C. Pedersen *et al*. [9] found that male cats, especially intact males, are not directly at risk of developing feline infectious peritonitis, as other authors claim [1].

Y. Yin *et al*. [10] report that most cats suspected for FIP belong to young animals and intact cats. The authors also found that the effusive form of infectious peritonitis is more common, namely in 85.8% of cats with suspected FIP.

Feline Infectious peritonitis has non-specific clinical symptoms. Thus, A. Kipar and M.L. Meli [11] state that anorexia, lethargy, weight loss, pyrexia, ophthalmic and neurological symptoms are non-specific for feline viral peritonitis.

According to recent studies, FIP has three main forms of course: effusive (wet), dry, and mixed. Therewith, A.M. Legendre *et al*. [12] established the median survival of cats for various forms of the disease. The effusive form of FIP is characterized by fluid accumulation in the abdominal and thoracic cavities – the median survival was 9 days in 21 cats with this form of the disease. The dry form of FIP is often characterized by pyogranulomatous infiltrates in the liver, kidneys, lymph nodes, eyes, and central nervous system. In the dry form, life expectancy is slightly higher and is within 1-200 days, which was found in 59 cats with FIP. In case of the mixed form, only 2 animals taking glucocorticosteroids were studied. Therewith, their life expectancy was 181 and 477 days, respectively.

The diagnosis of FIP is formed based on anamnesis data, results of physical examination, and general diagnostic procedures performed, as well as, if possible, data from post-mortem and microscopic examination of histopreparations. Therewith, classical indirect studies include morphological and biochemical blood tests with determination of the total content of whey protein, albumin and globulins, the value of the albumin/globulin ratio [12].

F. Mustaffa-Kamal *et al*. [13] noted the importance of T cells in the diagnosis of FIP. Thus, a decrease in the total number of white blood cells (WBC), lymphocytes, and T cells in the blood was observed during primary acute

infection for all experimental groups of animals with FIP. However, F. Riemer *et al*. [8] found that lymphopenia, which is much more common in the effusive form of FIP, was detected in only 26.8% of cats with a dry form.

E. Barker and S. Tasker [2] state that the clinical symptoms of feline infectious peritonitis can change over time, which often requires repeated clinical examinations. Non-specific clinical symptoms often increase and decrease, which is explained by the systemic inflammatory response, and are often found in cats with or without effusion. However, the absence of the above symptoms does not exclude feline infectious peritonitis. The authors also argue that routine clinical and laboratory studies in cats with FIP indicate the presence of a chronic systemic inflammatory response of the body, but these changes are not specific to the diagnosis of infectious peritonitis. That is why these studies are not considered informative. N. Safi *et al*. [14] conducted a haematological study of samples from nine cats with effusion and found that all animals had thrombocytopenia, hyperbilirubinemia, hyperglobulinaemia, and hypoalbuminemia. Three of these cats also had lymphopenia and jaundice.

J. Yousuf *et al*. [15] found that changes in indicators during biochemical blood tests in cats with FIP are variable and often non-specific, but there are several key abnormalities that doctors recommend considering before confirming the diagnosis. Thus, the above-mentioned authors indicate hyperglobulinaemia and hyperbilirubinemia. Hyperbilirubinemia occurs in 21-63% of cases of FIP and is more often observed in the effusive form, when the activity of the enzyme alanine aminotransferase (ALT), alkaline phosphatase (ALP) and γ-glutamyltranspeptidase is usually high (a moderate increase is also possible). In 89% of cases, hyperglobulinaemia is observed; often with hypoalbuminemia (detected in 64.5% of cases).

A more important diagnostic criterion for FIP is the determination of the ratio of albumin/globulin of blood or effusion. Thus, several variants of limit indicators were proposed that can potentially indicate the presence of feline infectious peritonitis. As a rule, this indicator is less than 0.4, which indicates a high probability of FIP [3].

Feline infectious peritonitis has always been considered the most common cause of death in young cats. Until 2019, it was also believed that feline infectious peritonitis is an incurable disease and ends in death [16].

Materials and Methods

The object of research was 12 cats with FIP aged from 6 months to 3 years, who were observed during 2021-2022. Among the cats under study, 8 had an effusive form of infectious peritonitis and 4 had a dry form. The study was performed based on the Department of Epizootology, Microbiology and Virology of the National University of Life and Environmental Sciences of Ukraine and the veterinary clinic "White Wolf" (Kyiv).

To establish a diagnosis, clinical and laboratory research methods were used, including morphological and biochemical blood tests. The haematological parameters were analysed in the veterinary clinic "White Wolf": for morphological studies, an automatic haematological analyser Mindray BC-5000 VET (China) was used, and an automatic analyser Goldsite GBA-1000 (China) was used to establish the parameters of biochemical parameters of blood serum. The data obtained were compared with a control group of clinically healthy cats (n = 12) who underwent a preventive examination in a veterinary clinic.

To establish a diagnosis of feline infectious peritonitis, cytological examination of exudate from the pleural and abdominal cavities and a Rivalta test were also performed.

For cytological examination, 2 ml of exudate was taken from the pleural and abdominal cavities into a test tube with an anticoagulant (test tubes with ethylenediaminetetraacetic acid were used). The use of anticoagulants prevents the formation of a clot, which complicates the reliable calculation of the cellular component of the exudate, as well as the possible inclusion of macrophages affected by feline coronavirus in the clot. Exudate from the thoracic and abdominal cavities in cats with an effusive form of infectious peritonitis was sampled under the control of an ultrasound machine (Mindray DP-10, China). T-FAST studies were performed using an ultrasound machine. In general, T-FAST studies in veterinary medicine are used to evaluate patients after acute blunt trauma or in the presence of pleural exudate. This study is performed in the left or right lateral position. Two examinations are conducted – transverse and longitudinal. The four fields of view are always evaluated, the condition of the lungs, heart, and the presence/absence of exudate are established.

After sampling the exudate to stabilize the biomaterial, the tube was turned over 6-7 times. Two smears were made on a slide. Each smear was left at room temperature to dry and then examined under a small and large magnification microscope (Optima Biofinder Bino 40x-1000x, Germany).

The Rivalta test is a rapid test for differential diagnosis of transudate from exudate. For its implementation, the Rivalta FIP-VETube diagnostic kit (Megacor, Great Britain) was used – a simple rapid test that allows quickly performing the necessary analysis. The essence of this method is that if there is a significant amount of protein in the exudate, a precipitate is formed when its drop is added to an aqueous solution of acetic acid. Before that, the test exudate was stained with methylene blue to evaluate the results of the study more accurately.

The obtained digital results were processed statistically, using Statistica 6.0 and Microsoft Excel 2019 software. Differences between the two indicators of the compared samples at $P < 0.05$, $P < 0.002$, and $P < 0.001$ were considered statistically significant.

Results and Discussion

In all cats under study, the first symptoms of feline infectious peritonitis were anorexia and lethargy. In 7 cats with an effusive form of FIP, an increase in abdominal volume was observed due to the accumulation of exudate. 1 cat had dyspnoea and compensatory tachycardia due to the accumulation of exudate in the thoracic cavity. Cats underwent ultrasound examination of the abdominal and thoracic cavities, which revealed the presence of a considerable amount of sonolucent exudate.

Among cats with a dry form of FIP, rapid weight loss was also observed. One cat had eye damage characterized by the development of keratitis and uveitis. According to research data from N.M. André *et al*. [1] a possible manifestation of feline infectious peritonitis may be the development

of rhinitis, but no such symptoms were found in 12 animals under study.

During abdominocentesis and pleurocentesis, straw-yellow, odourless, viscous fluid was aspirated (Fig. 1); in two cases, the liquid contained flakes of white fibrin.

Figure 1. Exudate from the abdominal cavity of a cat with feline infectious peritonitis. Viscous liquid, yellow in colour

The fluid from the pleural cavity also had a straw colour, a viscous consistency, without admixtures of fibrin (Fig. 2).

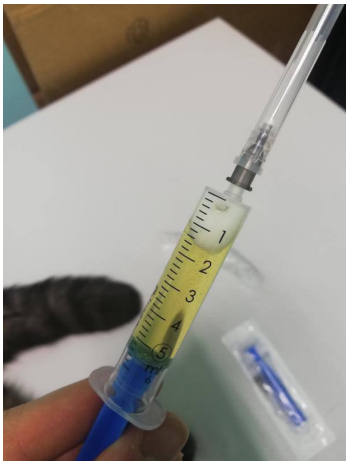

Figure 2. Exudate from the thoracic cavity of a cat with feline infectious peritonitis

During the Rivalta test in 5 out of 8 cats with an effusive form of FIP, the results were positive – sediment was formed during the study. That is, the predictive value of the Rivalta test for feline infectious peritonitis is 62.5%. According to S. Felten and K. Hartmann [3], the Rivalta test is affordable and fast to perform. Therefore, it can be recommended for the diagnostic protocol of examination of patients with exudate. According to these authors, this test shows sufficient sensitivity in the differential diagnosis of feline infectious peritonitis. If the test is negative, then other causes of exudate formation are much more likely than feline infectious peritonitis. However, the authors also claim that the predictive value of the Rivalta test for feline infectious peritonitis is only 66-81%.

To establish a diagnosis, cytological examination of exudate from the pleural and abdominal cavities was performed. Therewith, an elevated concentration of cells in the smears under study and the presence of red blood cells in the entire field of view were observed. Up to 60-70% of the cells found in the field of view were reactive macrophages of varying degrees of maturity (Fig. 3). Furthermore, activated macrophages with cytophagy were detected. Neutrophil phagocytosis and focal signs of emperipolesis were noted in two samples under study. In four samples, the presence of lymphocytes with many immunoblasts was observed, as well as small and medium-sized lymphocytes. Plasmocytes and neutrophils with a characteristic morphological structure were detected. In all 8 exudate samples under study, there was no pathological microflora.

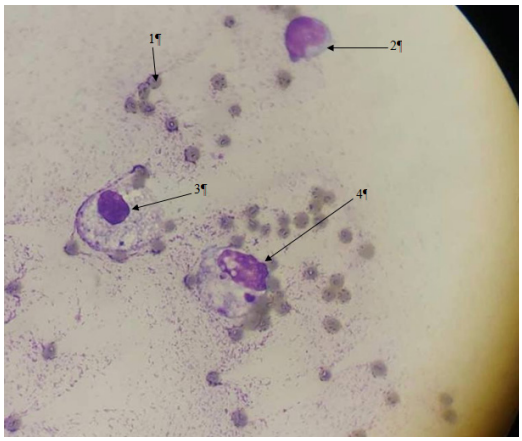

Figure 3. Microscopic picture of exudate from the abdominal cavity in a cat with diagnosed feline infectious peritonitis

Notes: 1 – red blood cells; 2 – T-lymphocyte; 3 – macrophage; 4 – macrophage with signs of phagocytosis.

In cats with a dry form of FIP, ultrasound diagnostics revealed inflammatory processes in the pleural and abdominal cavities, an increase in mesenteric lymph nodes. In two sick animals, a small amount of exudate was found between the intestinal loops.

To confirm the diagnosis, several laboratory tests were performed, including morphological and biochemical analysis of venous blood. Therewith, a range of indicators that are generally accepted in veterinary clinics and veterinary laboratories was determined [16].

During a morphological blood test, 12 cats were diagnosed with non-generative anaemia and lymphopenia, which is probably associated with the chronic course of the disease. Two animals were found to develop thrombocytopenia with a platelet count of 192 × 10^9/l at the reference rate of 200-600 × 10^9/l.

Notably, due to the development of anaemia in animals, a decrease in the haematocrit value was observed. In one cat, the number of red blood cells was 3.4 × 10^{12}/l, and the haematocrit value is 26% with reference values of 4.6-10 × 10^{12}/l and 36-55%, respectively. Therewith, the specified animal needed a blood transfusion.

Table 1 presents the results of a morphological blood test in 12 cats with feline infectious peritonitis and a similar number of cats in the control group.

Table 1. Morphological parameters of cat blood for feline infectious peritonitis (M ± m, n = 12)

Indicator	Unit of measurement	Experimental group of animals		Control group	Reference values
Red blood cells (RBC)	$x10^{12}$/l	4.27 ± 0.71*	↓	6.4 ± 0.32	4.6-10
Haemoglobin	g/l	97.7 ± 9.02	N	137.0 ± 10.12	93-153
ESR	mm/h	27.78 ± 11.13	N	11.0 ± 1.39	0-22
Haematocrit (HTC)	%	34.02 ± 3.74*	↓	50.0 ± 2.25	36-55
White blood cells (WBC)	$x10^9$/l	14.98 ± 1.38	N	13.2 ± 0.76	4.5-15.5
Juvenile neutrophils	$x10^9$/l	0	N	0	0
Band neutrophils	$x10^9$/l	0.21 ± 0.12	N	0.1±0.1	0-0.4
Segmented neutrophils	$x10^9$/l	10.76 ± 2.55	N	5.39 ± 3.19	2.5-12.5
Nuclear shift	juvenile+band/ segmented	0.02 ± 0.05	N	0.02 ± 0.01	0-0.08
Eosinophils (EO)	$x10^9$/l	0.6 ± 0.17	N	1.0 ± 0.02	0-1.6
Basophils (BA)	$x10^9$/l	0.04 ± 0.01	N	0	0-0.04
Lymphocytes (LYM)	$x10^9$/l	1.32 ± 0.62**	↓	6.0 ± 1.03	2.3-7.0
Monocytes (MON)	$*10^9$/l	0.69 ± 0.32	N	0.09 ± 0.25	0-1.9
Platelets (PLT)	$*10^9$/l	252.0 ± 67.56	N	497.0 ± 28.73	200-600

Note: * P < 0.05; * * P < 0.001, significant compared to the control group. ↓ – reduction of the indicator value; N – corresponds to the control limits

In general, morphological examination of the blood of patients with feline infectious peritonitis did not reveal specific changes that would be inherent in this particular disease. Based on the research results presented in Table 1, in the blood of cats with FIP, a 1.5-fold decrease in the number of red blood cells (P < 0.05) was observed, and the number of lymphocytes decreased 4.5-fold (P < 0.002) compared to the control group of cats. Anaemia and lymphopenia can also occur in diseases such as panleukopenia, chronic viral infections (feline viral immunodeficiency, viral leukaemia of cats), neoplasia, feline autoimmune haemolytic anaemia, etc. [4].

During the biochemical study of blood serum, special attention was paid to determining the level of total protein, albumin, globulin, bilirubin, and the activity of liver-specific enzymes – alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (AP) and γ-glutamyltranspeptidase (γ-GTP).

According to N.C. Pedersen *et al*. [9], it is these indicators that are prognostic of FIP in most cases. However, the authors do not believe that their research allows confirming or refuting this diagnosis.

The results of a biochemical study of the blood serum of 12 cats with feline infectious peritonitis in comparison with the results of the control group of cats are presented in Table 2.

Table 2. Results of biochemical examination of cat blood serum with feline infectious peritonitis (M ± m, n = 12)

Indicator	Unit of measurement	Experimental group of animals		Control group	Reference values
Total protein (TP)	g/l	84.50 ± 7.28	N	76.0 ± 2.48	59-89
Albumin (ALB)	g/l	19.77 ± 1.71*	↓	31.0 ± 3.85	22-39
Globulin (GLOB)	g/l	62.50 ± 3.88*	↑	45.0 ± 4.21	21-53
Albumin/globulin ratio	–	0.36 ± 0.04***	↓	0.7 ± 0.05	< 0.4 – probable FIP 0.4-0.8 – possible FIP > 0.8 – improbable FIP
Creatinine (CREA)	μmol/l	89.39 ± 10.05	N	129.0 ± 12.90	43-165
Urea (UREA)	mmol/l	6.36 ± 0.78	N	9.8 ± 2.78	4-12.9
Total bilirubin (TBIL)	μmol/l	25.80 ± 8.82*	↑	4.0 ± 2.27	0-11
Direct bilirubin (DBIL)	μmol/l	8.80 ± 7.37*	↑	0	0-8
Alanine aminotransferase (ALT)	IU/l	40.07 ± 6.16	N	65.0 ± 5.34	10-85
Aspartate aminotransferase (AST)	IU/l	82.03 ± 24.02	↑	47.0 ± 3.19	10-56
Glucose (GLUC)	mmol/l	6.29 ± 0.39	N	4.9 ± 1.12	3.4-7.9
Alkaline phosphatase (AP)	IU/l	103.42 ± 8.12	↑	97.0 ± 1.74	6-102
GGT	IU/l	4.70 ± 1.50	N	6.0 ± 2.17	1-10

Note: * P < 0.05; **P < 0.002; *** P < 0.001, significant compared to the control group. ↓ – decrease in the indicator value; ↑ – increase in the indicator value; N – corresponds to the control limits

All 12 cats with feline infectious peritonitis showed a substantial increase in content of total and direct bilirubin levels in blood serum. Thus, the level of total bilirubin in cats of the experimental group increased by 6.5 times ($P < 0.05$) compared to the same indicator in cats of the control group. Notably, with such values of these indicators, icterus of the mucous membranes was observed only in 5 cats with FIP.

At the same time, one cat was found to have hypoglycaemia, the serum glucose concentration was 2.9 mmol/l at the reference values of 3.4-7.9 mmol/l, and two cats were found to have hyperglycaemia – the glucose level corresponded to the values of 8.8 and 9.1 mmol/l, respectively. Probably, this increase in serum glucose concentration is associated with the stressful state of cats. Therewith, in 11 cats, relative kidney-specific indicators (creatinine and urea concentrations) corresponded to the limits of reference values, while in one cat an increase in the value of these indicators was observed (creatinine – 210 mmol/l at reference values of 43-165 mmol/l; urea – 15.8 mmol/l at reference values of 4-12.9 mmol/l).

Hypoalbuminemia was observed in all the cats under study, with simultaneous development of hyperglobulinaemia. Therewith, the level of total protein in the blood serum stayed within the reference values.

The albumin/globulin ratio in the blood serum of experimental cats was also determined. Thus, in 11 sick cats, its value was < 0.4, and in one cat – 0.46. Notably, in the control group of cats that are clinically healthy, the albumin/globulin ratio in the blood serum was kept within the reference values. In the experimental group of cats, the albumin/globulin ratio in blood serum was characterized by values almost 2.0 times lower ($P < 0.001$) than in the control group of animals.

S. Felten and K. Hartmann [3] reported a 92% prognostic positive result of an albumin/globulin ratio < 0.8 based on a theoretical population with a 75% incidence of feline infectious peritonitis.

N.C. Pedersen [4] found that a low albumin/globulin ratio has a high predictive value in cats with severe clinical symptoms of feline infectious peritonitis. Furthermore, it was found that when the number of FIP cases is low, the albumin/globulin ratio is useful for excluding FIP upon differential diagnostics. However, it is not predictive for establishing this diagnosis.

According to the conducted studies, the predictive value of a positive result < 0.4 albumin/globulin ratio is 91.6%. When determining the predictive value of a positive result of the albumin/globulin ratio to the Hartmann level (< 0.8), the predictive value in the conducted study is 100%.

Having analysed the results of a biochemical blood test of cats with feline infectious peritonitis, it can be concluded that changes in these indicators are not specific.

The level of albumin, globulin, and their ratio should be considered important in the diagnosis of this disease. But these indicators are insufficient to focus on them exclusively. To diagnose feline infectious peritonitis, it is necessary to conduct a complex of clinical and laboratory tests. It is necessary to find the albumin/globulin ratio not only in the blood serum, but also in the effusion of the effusive form of feline infectious peritonitis.

According to N.C. Pedersen, M. Perron, M. Bannasch, serological studies (immunoenzyme and immunochromatographic tests) are not prognostic if they are used in monomode [4; 5; 9].

Thus, serological studies should be used only to monitor the condition of the animal with the development of coronavirus infection, and they are impractical for diagnosing feline infectious peritonitis.

In modern-day conditions, the diagnosis of feline infectious peritonitis is considered confirmed only after an immunohistochemical study of biopsies. But in practice, cats with infectious peritonitis often have a serious condition that makes anaesthesia and sampling impossible. Notably, in Ukraine, there are no veterinary laboratories that can conduct an immunohistochemical study of a biopsy to identify the causative agent of feline infectious peritonitis. That is why, in present-day realities, the diagnosis of feline infectious peritonitis is found by excluding other differential diagnoses.

Conclusions

The study of the problematic issue of establishing a diagnosis for feline infectious peritonitis has shown that the diagnosis of this disease should be comprehensive and include haematological studies.

In case of feline infectious peritonitis, changes in the morphological composition of blood and biochemical parameters are non-specific. Morphological examination of the blood of cats with infectious peritonitis revealed the development of lymphopenia, non-generative anaemia, and a decrease in the haematocrit value.

Biochemical parameters such as the determination of globulins, albumin, and bilirubin in the blood serum are of diagnostic importance for feline infectious peritonitis. Cats with infectious peritonitis have a decrease in albumin levels and an increase in globulins. Therewith, the total protein content stays within the reference limits.

The Rivalta test is a fast research method, but it only allows distinguishing exudate from transudate. Its prognostic value for infectious peritonitis is only 62.5%. Therefore, the diagnosis of feline infectious peritonitis cannot be based solely on this method. However, it should be included in the diagnostic protocol.

Diagnostic value for feline infectious peritonitis is the albumin/globulin ratio in the blood serum and in the effusion fluid (for the effusive form of infectious peritonitis). The predictive value of a positive result < 0.4 of the albumin/globulin ratio is 91.6%, and the predictive value of a positive result < 0.8 is 100%. Determination of the albumin/globulin ratio should not be used as the sole parameter for the diagnosis of feline infectious peritonitis.

Further scientific research should be aimed at investigating the clinical features of the course of feline infectious peritonitis and finding effective methods for its laboratory diagnosis. A critical issue is also the improvement of treatment measures for the development of this disease.

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Морфологічні та біохімічні зміни показників крові в котів з інфекційним перитонітом

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Анотація. Інфекційний перитоніт котів – це захворювання котячих, збудником якого є мутант коронавірусу, що призводить до загибелі молодих тварин. На сьогодні цю патологію вважають невиліковною, а тому вона потребує детального вивчення. Метою дослідження було встановлення особливостей клінічного прояву та гематологічних показників у котів за інфекційного перитоніту. Представлено результати клініко-лабораторного дослідження 12 котів, віком від 6 місяців до 3 років, у яких було діагностовано інфекційний перитоніт ефузивної та сухої форм. У роботі використовували клінічні, лабораторні та візуальні методи дослідження. Лабораторний аналіз включав дослідження морфологічних і біохімічних показників крові, цитологічне дослідження випоту з абдомінальної й плевральної порожнин, пробу Рівальта. В результаті цитологічного дослідження випоту із

зазначених порожнин виявлено високу концентрацію клітин, скупчення макрофагів, фагоцитоз нейтрофілів, еритроцити в усьому полі зору. Встановлено, що прогностичність проби Рівальта за інфекційного перитоніту котів становить 62,5 %. За гематологічного дослідження в усіх хворих тварин діагностовано анемію, лімфопенію, гіпоальбумінемію, гіперглобулінемію, гіпербілірубінемію, підвищення активності відносноспецифічних для печінки ферментів (аспартатамінотрансферази, лужної фосфатази). У двох тварин відзначали тробмоцитопенію, а в одного кота встановлено підвищення в крові маркерів функціонального стану нирок (креатиніну, сечовини). Важливим діагностичним показником слід також вважати альбумін/глобулінове співвідношення в сироватці крові. Так, за інфекційного перитоніту котів цей показник має бути < 0,4. У 11 хворих котів це співвідношення характеризувалося значеннями нижчими за 0,4, а у однієї тварини цей показник становив 0,46. В цілому, отримані дані морфологічного та біохімічного аналізу крові не є специфічними для інфекційного перитоніту котів, а тому з діагностичною метою рекомендуємо їх комплексне дослідження. Проведена об'єктивна оцінка доступних методів лабораторної діагностики сприятиме створенню діагностичного протоколу за інфекційного перитоніту котів

Ключові слова: коронавірусна інфекція, клінічні та гематологічні дослідження, проба Рівальта, цитологічне дослідження

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Pathohistological Changes in Aborted Foetuses of Cows Due to Neosporosis: Evidence from Ukraine

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Abstract. Neosporosis is a parasitic disease characterized by abortions and the birth of weak offspring in cows. The causative agent of *Neospora caninum* is an obligate, protozoan parasite that belongs to the type *Apicomplexa*. The relevance of the study is conditioned upon the adverse impact of neosporosis on the economy of Ukraine (loss of productivity, veterinary and diagnostic costs). Furthermore, the issue of neosporosis is understudied. In this regard, the purpose of this study was to establish pathohistological changes in aborted foetuses and the foetal part of placentas and to confirm the involvement of the parasite (*Neospora caninum*) in cases of abortions recorded in different regions of the country. Two methods were used to investigate this problem: histological and real-time polymerase chain reaction. In aborted foetuses positive for *N. caninum*, the following pathohistological changes were most often detected: focal gliosis and perivascular mononuclear infiltrates in the brain; focal or diffuse mononuclear infiltration in the heart and skeletal muscles; periportal mononuclear infiltrates in the liver; focal necrosis of the mucous membrane and mononuclear infiltration in the foetal part of the placenta. Changes were less often detected in the lungs – mononuclear infiltration of the interstitium and diffuse lymphocytic alveolitis, and in the kidneys – diffuse interstitial mononuclear infiltration. No changes were found in the spleen. Neospore-like cysts were found in one out of twelve foetuses. Lesions established of foetal organs and placentas are inherent in neosporosis. The results of histological studies substantially complement the data of other authors, confirm the involvement of *N. caninum* in the occurrence of abortions in cows in certain regions of Ukraine, and also represent practical value for the diagnosis and control of neosporosis in cattle

Keywords: cattle, *Neospora caninum*, abortion, histology

Introduction

Neospora caninum is a unicellular parasite that causes neosporosis, a parasitic disease that results in reproductive problems, namely abortions in cows [1-3]. Furthermore, the disease is widespread in the world, as evidenced by a meta-analysis conducted by Tooran Nayeri *et al.*, [4] and about 3,000 scientific papers on this topic [5].

The parasite was first detected in the brain and skeletal muscle of Boxer puppies in Norway in 1984 [6] and described in 1988 [7]. The causative agent *N. caninum* was first isolated from aborted foetuses of cows in 1993 [8].

Dogs, coyotes, wolves, and dingoes have been identified as definitive hosts of *N. caninum*, while cattle, deer, sheep, buffalo, bison, rodents, birds, and horses are intermediate hosts [9]. In cows, infection with the parasite can occur from mother to foetus, and in the postnatal period – due to ingestion of sporulated oocysts [10; 11].

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Pathohistological changes that most often occur in foetuses and placentas infected with *N. caninum* include periportal hepatitis and multifocal hepatocellular necrosis; myocarditis and pericarditis; perivascular infiltrates, focal necrosis and foci of gliosis in the brain; non-purulent myositis; focal necrosis and non-purulent placentitis [12-14]. These changes do not have a clear localization and correspond to different severity of the lesion [15-17].

Ahmad Nematollahi *et al*. described similar pathohistological changes in infected foetuses: acute congestion, perivascular and perineuronal oedema, spongiosis, perivascular infiltrates, focal gliosis and necrosis were noted in the brain and spinal cord; in the placenta – vasculitis, acute congestion, perivascular infiltration with mononuclear cells, vascular thrombosis, focal placentitis and necrosis in the cotyledons; in the heart – focal pericarditis and focal haemorrhages [18].

Amir Kamali *et al*. investigated 56 brain samples of aborted foetuses, of which 16 (28%) showed non-purulent encephalitis inherent in neosporosis. Acute hyperaemia (100%), oedema (100%), gliosis (93%), perivascular infiltrates (63%), haemorrhages (51%), focal necrosis (25%), non-purulent meningitis (5%), and satellitosis (2%) were also noted in this study [19].

In the brain, heart, liver, lungs, kidneys of 34 foetuses infected with *N. caninum*, histological studies by Esther Collantes-Fernández *et al*. established multiple centres of non-purulent infiltrates with multifocal necrotic areas surrounded by inflammatory cells. According to their data, the affected organs were the heart, liver, and brain, less affected – the lungs and kidneys [20].

As a result of statistical analysis of the published data, it was found that in 85% of cases, pathological changes in aborted foetuses are mainly localized in the central nervous system [21]. Furthermore, researchers from Brazil and Greece emphasize the influence of the parasite on the reproductive system, which adversely affects the reproduction rates of cattle [22; 23].

Neosporosis causes significant economic losses both at the level of individual livestock farms and in the entire livestock industry due to the deterioration of reproduction indicators, reduced productivity and the costs associated with preventive measures [24-26]. This disease is difficult to control since there is no specific treatment and affordable and efficacious vaccine prevention.

In Ukraine, there are laboratory-confirmed cases of abortions caused by *N. caninum* [27] and, since there are only isolated reports on this issue, *the purpose of this study* is to evaluate pathohistological changes in aborted foetuses and the foetal part of placentas and to confirm the involvement of *N. caninum* in recorded cases of cow abortions in Ukraine.

Materials and Methods

Aborted foetuses and/or foetal placentas from cows were selected as samples for the study. As a result, 13 samples were taken, of which two were incomplete: one had no placenta, and the other had no foetus.

Aborted foetuses and the foetal part of placentas were obtained from farms in Cherkasy (3 samples), Kyiv (3 samples), Khmelnytskyi (4 samples), Sumy (1 sample), Chernihiv (1 sample), and Ternopil (1 sample). The term of pregnancy at which the abortion occurred was set according to the data specified in the attached documents.

The selection of samples for genetic, molecular, and histological research was performed directly in the section hall of "Veterinary Diagnostics Centre" LLC (Kyiv) during the autopsy.

The selection of samples for genetic, molecular, and histological research was performed directly in the section hall of "Veterinary Diagnostics Centre" LLC (Kyiv) during the autopsy. For this, the brain, heart, lungs, spleen, liver, kidneys, skeletal muscles of the neck, and cotyledons of the placenta were selected. Pieces sized 1-1.5 cm were cut from the organs under study, at the border of normal and pathologically changed tissue.

To fix pathological material, pieces of organs were transferred to 10% buffered formalin (Leica, Germany) and kept in it for at least 48 hours. After fixation, pieces sized 0.5×0.5 mm were cut from the selected organs with a blade (considering the specific features of the structure of each organ and the presence of visible pathological changes) and placed in plastic cassettes, which were labelled according to the assigned number. Next, primary processing of the material (dehydration in ethyl alcohol and sealing in liquid paraffin) was performed using a histoprocessor STP-120 (Microm, Germany). The duration of primary tissue treatment was 12 hours [28].

To produce paraffin blocks, a station for filling tissues with paraffin EC 350 (Microm, Germany) was used. For this, tissue samples were transferred to special metal moulds and filled with hot paraffin. After cooling the paraffin on cryoconsole, the resulting paraffin blocks were cut on a rotary Microtome HM 340E (Microm, Germany), while the cut thickness was 4 µm. The sections were transferred to slides and dried in a thermostat for 2 hours at 47°C. After drying, they were stained with haematoxylin and eosin (Leica, Germany) according to the generally accepted staining method, and placed in a fixing medium for the manufacture of permanent preparations (Thermo Fisher, USA) [28; 29].

Histological analysis of all samples was performed at magnifications of x100, x200, x400, and x1000 using an Axioskop 40 light microscope (Carl Zeiss, Germany), W-PI 10x/23 glasses, and 10, 20, 40, 100 objectives. Canon PC 1049 Power Shot G5 camera, 5.0 MegaPixels through Adapter tube BAYONET-52 mm (EASY FIT) for Canon G3 (Canon, Japan) was used for photo registration.

In all the samples under study, prior to histological examination, *N. caninum* DNA was detected by polymerase chain reaction. The amplification reaction was carried out using commercial test kits of European manufacturers on QuantStudio 5 Real Time PCR System equipment (Thermo Fisher Scientific, USA), following the manufacturer's recommendations.

Results and Discussion

Abortions in cows were registered: at 4 months of gestation – five samples, at 5 months – three, at 6 months – four, and at 7 months – one (Table 1).

Table 1. The main pathohistological changes in aborted foetuses and the foetal part of the placenta of cows, considering the pregnancy period

Duration of pregnancy	Number of samples	Pathohistological changes	Number of affected samples	Percentage of affected samples, %
Month 4	5	Encephalitis	4	80
		Myocarditis	4	80
		Myositis	5	100
		Hepatitis	3	60
		Placentitis	4	80
Month 5	3*	Encephalitis	1	50
		Myocarditis	2	100
		Myositis	1	50
		Hepatitis	2	100
		Placentitis	2	67
Month 6	4**	Encephalitis	3	75
		Myocarditis	4	100
		Myositis	3	75
		Hepatitis	3	75
		Placentitis	2	67
Month 7	1	Encephalitis	1	100
		Myocarditis	1	100
		Hepatitis	1	100
		Placentitis	1	100

Note: * – one foetus is missing; ** – one placenta is missing

Focal gliosis (Fig. 1) and perivascular mononuclear infiltrates were found in the brain of six foetuses, and perivascular, perineuronal oedema and perivascular mononuclear infiltrates were found in two. In other foetuses, multiple sites of necrosis, perivascular mononuclear infiltrates, single necrotic neurons, focal necrosis, and perivascular mononuclear infiltrates in its soft shell were also noted in the brain.

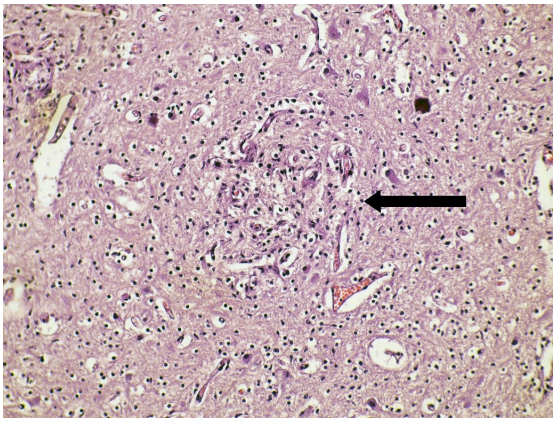

Figure 1. Focus of gliosis in the grey matter of the foetal brain (arrow). Haematoxylin-eosin, ×200

In the hearts of five foetuses, diffuse mononuclear infiltration in the pericardium (Fig. 2), myocardium, and endocardium was recorded, in two – diffuse mononuclear infiltration in the pericardium and myocardium, in four – diffuse mononuclear infiltration in the myocardium (Fig. 3).

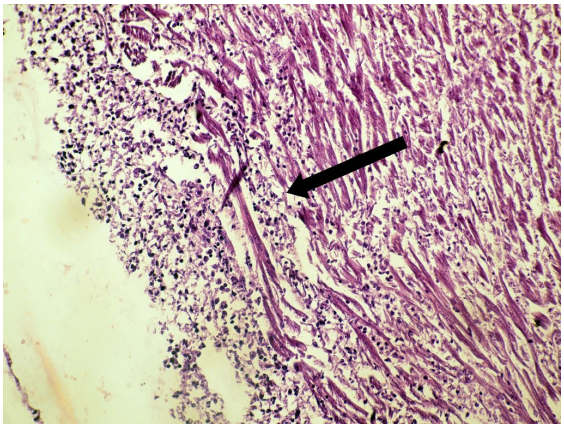

Figure 2. Diffuse mononuclear infiltration in the foetal pericardium (arrow). Haematoxylin-eosin, ×200

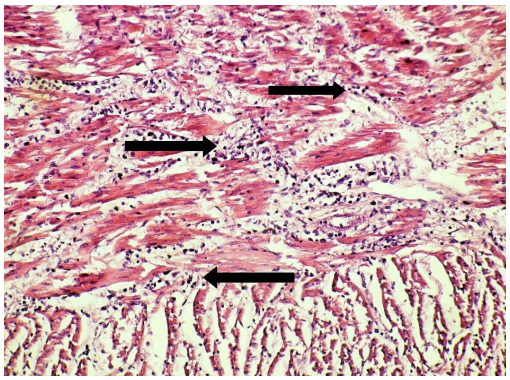

Figure 3. Diffuse mononuclear infiltration in the foetal myocardium (arrows). Haematoxylin-eosin, ×200

Mononuclear infiltration of the interstitium was found in the lungs of two foetuses, focal desquamation of the epithelium of the bronchi and bronchioles, and diffuse lymphocytic alveolitis in another two. Therewith, periportal mononuclear infiltrates were noted in the liver of nine foetuses (Fig. 4), in three – focal necrosis of hepatocytes and in two – focal dystrophy of hepatocytes.

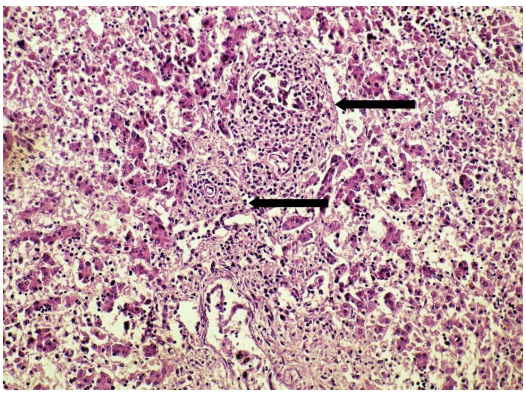

Figure 4. Periportal mononuclear infiltrates in the foetal liver (arrows). Haematoxylin-eosin, ×200

No changes were found in the spleen of twelve foetuses. The skeletal neck muscles of seven foetuses were characterized by diffuse mononuclear infiltration (Fig. 5), for two – focal mononuclear infiltration.

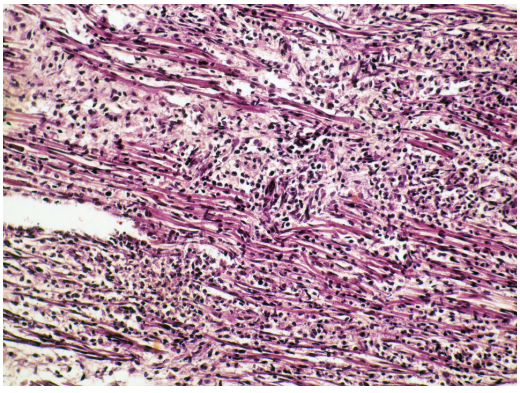

Figure 5. Diffuse mononuclear infiltration in the skeletal muscle of the foetal neck. Haematoxylin-eosin, ×200

Single neosporous cysts and focal mononuclear infiltrates were found in the tongue muscles of one foetus (Fig. 6).

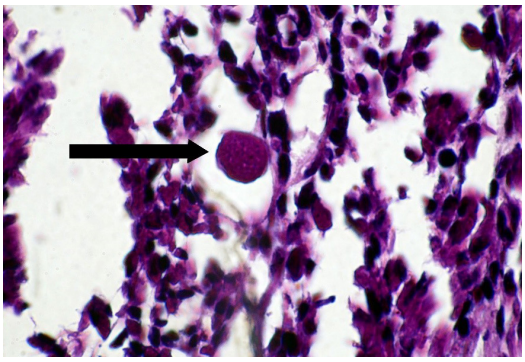

Figure 6. Neosporous cyst in the foetal tongue muscles (arrow). Haematoxylin-eosin, ×1000

Furthermore, diffuse interstitial mononuclear infiltration was diagnosed in the kidneys of three foetuses (Fig. 7), and focal necrosis in one foetus.

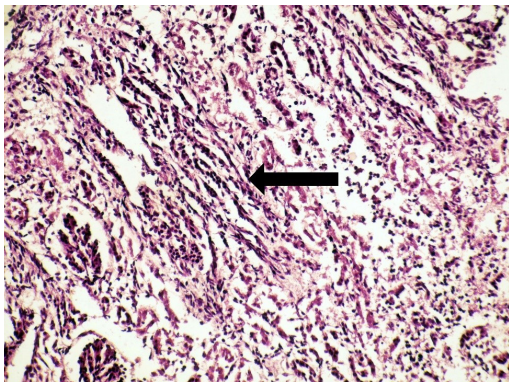

Figure 7. Diffuse interstitial mononuclear infiltration in the foetal kidney (arrow). Haematoxylin-eosin, ×200

In five foetal placentas, focal necrosis of the mucous membrane was established, in three – fibrin deposition on the mucous membrane, and in nine more – focal mononuclear infiltration.

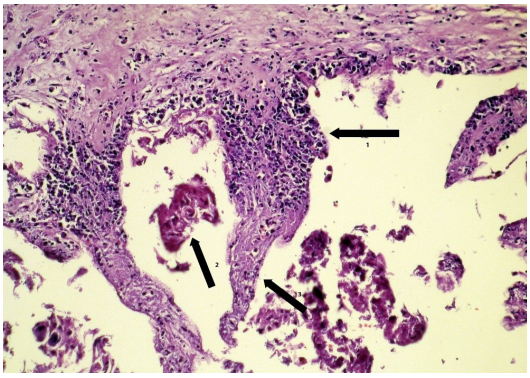

Figure 8. Focal mononuclear infiltration (Arrow 1), deposition of fibrin on the mucous membrane (Arrow 2) and focal necrosis of the mucosal villi (Arrow 3) of the foetal part of the placenta (cotiledon) of the foetus. Haematoxylin-eosin, ×200

Thus, in general, encephalitis was diagnosed in 9 foetuses, meningitis – in 1, pericarditis – in 7, myocarditis – in 11, endocarditis – in 5, myositis – in 9, hepatitis – in 9, pneumonia – in 4, nephritis – in 3, and placentitis – in 9 (Fig. 9).

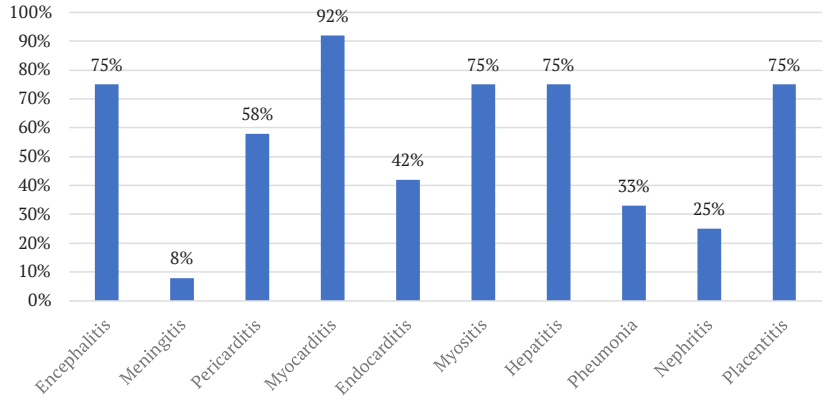

Figure 9. Pathohistological changes in aborted foetuses and foetal part of cow placentas

Regardless of the term of pregnancy, aborted foetuses were mainly diagnosed with encephalitis, myocarditis, myositis, hepatitis, and placentitis (Table 1).

Pathohistological changes (encephalitis, myositis, myocarditis, hepatitis, placentitis), which were detected in most of the foetuses and the foetal part of placentas under study (Table 1), are characteristic of infection caused by *N. caninum* and are consistent with the results of similar studies [12; 13; 17]. Pericarditis, endocarditis, pneumonia, nephritis, and meningitis were somewhat less common. Mononuclear infiltration was present in all organs except the spleen. There were also no changes in the spleen of 12 foetuses and no reports describing them, although the organ itself was selected for research.

The changes mentioned above are non-specific. They are also noted for Bovine viral diarrhoea virus (BVDV) and infectious bovine rhinotracheitis (IBR) in an aborted foetus [32; 33].

Single neosporous cysts were found in only 1 out of 12 foetuses. These results correspond to the results of other researchers, where tissue cysts of *N. caninum* were found extremely rarely during histological examination in foetuses and placentas [20; 30]. Furthermore, the established single neosporous cysts were localized in the muscles of the tongue, while other researchers usually noted them in the brain [15; 30; 34].

Pathohistological changes noted in various organs and systems of aborted foetuses may indicate that the infection caused by *N. caninum* has a systemic manifestation.

Dependence and significant differences between the detected pathohistological changes and the duration of pregnancy at which abortion occurred in cows were not noted. As for the duration of pregnancy, abortions in cows were recorded mainly in the period from 4 to 6 months, although they can also occur from 3 to 9 months of pregnancy [17; 20].

The observed characteristic changes confirm the involvement of *N. caninum* in abortions in cows in livestock farms of Cherkasy, Kyiv, Khmelnytskyi, Sumy, Chernihiv, and Ternopil regions of Ukraine.

The study applied two research methods. First, the polymerase chain reaction as the most accurate and sensitive method. However, it is not sufficient to confirm the role of *N. caninum* in abortion since there is a high probability of participation or complicity of other factors that can provoke abortion. The histological method allowed identifying changes inherent in neosporosis, but in the absence of specific changes, it cannot be said that they are caused by *N. caninum*. These methods are successfully used in obstetric practice to find the causes of abortions in cows [31]. Therefore, to establish a final diagnosis, the authors of this study recommend the comprehensive use of two research methods at once, as this affects the speed of decision-making and the organization of measures to prevent the spread of neosporosis and its consequences.

Conclusions

It was found that abortions in cows with neosporosis are mainly recorded in the second trimester of pregnancy. Most frequently, pathohistological changes are detected in the brain, heart, skeletal muscles of the neck, liver, and the foetal part of the placenta. Basically, diffuse mononuclear infiltration is noted in the organs of aborted foetuses and the foetal part of placentas of cows. Therewith, as a result of the conducted studies, neosporosis was confirmed as one of the causes of abortions in cows from six regions of Ukraine. It was found that the histological method of examination cannot be used in isolation to establish a definitive diagnosis, since pathohistological changes in neosporosis are non-specific and may be misinterpreted. Therefore, to diagnose this disease in animals, it is recommended to combine two methods: polymerase chain reaction and histological studies.

The results obtained can be used to improve the laboratory diagnosis of neosporosis in cows, control its spread in livestock farms, and develop instructions for further prevention of this disease.

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Патогістологічні зміни в абортованих плодів корів за неоспорозу в Україні

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Анотація. Неоспороз – паразитарна хвороба, яка у корів характеризується абортами та народженням слабкого потомства. Збудник *Neospora caninum* – облігатний, найпростійший паразит, який відноситься до типу *Apicomplexa*. Актуальність дослідження зумовлена негативним впливом на економіку (втрата продуктивності, ветеринарні та діагностичні витрати) та недостатнім дослідженням неоспорозу в Україні. У зв'язку з цим, метою роботи було встановлення патогістологічних змін у абортованих плодів і плодової частини плацент та підтвердження участі паразита (*Neospora caninum*) у випадках абортів, які зафіксовано в різних областях країни. Для дослідження цієї проблеми використовували два методи: гістологічний та полімеразної ланцюгової реакції в реальному часі. У позитивних щодо *N. caninum* абортованих плодів найчастіше виявляли такі патогістологічні зміни: осередковий гліоз і периваскулярні мононуклеарні інфільтрати в головному мозку; осередкову або дифузну мононуклеарну інфільтрацію в серці та скелетних м'язах; перипортальні мононуклеарні інфільтрати в печінці; осередковий некроз слизової оболонки та мононуклеарну інфільтрацію у плодовій частині плацент. Рідше зміни виявляли в легенях – мононуклеарна інфільтрація інтерстиції й дифузний лімфоцитарний альвеоліт, та нирках – дифузна інтерстиціальна мононуклеарна інфільтрація. У селезінці змін не встановлено. Неоспороподібні цисти виявлені в одному з дванадцятьох плодів. Встановлені ураження органів плодів і плацент є характерними для неоспорозу. Результати гістологічних досліджень істотно доповнюють дані інших авторів, підтверджують причетність *N. caninum* до виникнення абортів у корів окремих областей України, а також становлять практичну цінність для діагностики та здійснення контролю за неоспорозом у великої рогатої худоби

Ключові слова: велика рогата худоба, *Neospora caninum*, аборт, гістологія

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Efficacy of Decoquinate in Pelleted Feed in Case of Eimeriosis in Lambs

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Abstract. Eimeriosis of sheep reduces the profitability of the production of wool, leather, meat, and milk, which causes substantial damage to the sheep industry, and therefore requires the introduction of effective means of prevention and treatment of animals from this infestation. The purpose of this study was to establish the efficacy of decoquinate in granular feed in case of lamb eimeriosis. As a result of coproscopic studies according to DSTU 5079 2008 "Veterinary medicine. Methods of laboratory diagnosis of eimerioses" determined the extent of invasion of lambs by oocysts of various Eimeria species, namely *E. arloingi* – 41%, *E. crandallis* – 29%, *E. intricate* – 15%, *E. faurei* – 15%, which are registered in animals of separate farms of the Cherkasy region. Clinical symptoms of eimeriosis are observed in young animals from the age of one month and are characterized by anaemia of visible mucous membranes, diarrhoea, depression of the general condition, weakness, and predominantly acute course of invasion with high mortality. The manifestation of clinical symptoms of the disease depends on many factors, namely the age of lambs, the sanitary situation on the farm, changes in feed fattening programs, stressful situations that arise due to the movement of sheep to other premises or weaning lambs from ewes. In older lambs, as a rule, the disease has a chronic and asymptomatic course. In such animals, Eimeria oocysts are excreted in faeces in much smaller quantities than in one-month-old lambs during an acute course of infection. The maximum values of invasion extensiveness (100%) and invasion intensiveness (12,000 oocysts in 1 g of faeces) were found in two-month-old lambs. Invasion in the digestive canal of these animals worsens the absorption of feed nutrients, which contributes to a decrease in the average daily weight gain (on average 155.3 g/day), which increases with treatment of animals with decoquinate (on average 185.7 g/day). In general, the use of decoquinate in sick lambs at a dose of 1 mg/kg of body weight for 28 days shows prominent therapeutic and preventive effectiveness. Therewith, extensefficiency is 90%, and parasite carrier is observed only in 10% of animals. Therefore, for the effective treatment of lambs of distinct age groups, the decoquinate preparation can be recommended, which is especially important for farms with intensive rearing of young sheep

Keywords: oocysts, Eimeria, invasion, treatment, diagnostic methods

Introduction

Eimeriosis is a disease caused by single-celled protozoan parasites of the genus *Eimeria* which can affect all types of domestic ruminants (cattle, bison, sheep, goats, deer). Each type of livestock is infected with species varieties of *Eimeria* spp. This shows their specificity for parasitism. Since 1902, Eimeria was considered non-pathogenic, but by the 1960s they were recognized as causing animal diseases. Furthermore, infected animals are vulnerable to various pathogens of infections and infestations. [1].

On the territory of Ukraine, according to research by scientists [1; 2], Eimeriosis is a common disease in sheep, where pathogens *E. arloingi, E. crandallis*, *E. intricate*, and

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E. faurei occupy one of the leading places. Given that young animals usually get sick [2; 3] and the invasiveness is 99%, then parasitic diseases, namely Eimeriosis, cause considerable economic losses to farms and their owners, reducing the profitability of sheep breeding by 30%.

Nowadays, sheep breeding is one of the fields of activity of farmers in the central part of Ukraine. The problem for most farms and homesteads is non-observance of general sanitary and hygienic measures, which leads to losses in this area of animal husbandry and adversely affects zootechnical and economic indicators of profitability, which restrains the increase in the liquidity of sheep breeding [3].

When raising lambs, farmers strive for a stable increase in the body weight of the animals. This can only be achieved when the animals receive sufficient feed and when farmers pay due attention to the health of the lambs [4]. For farmers all over the world, the lack of desired results due to the death of lambs from pathogens of eimeriosis is noticeable [5; 6;].

To ensure the proper effect in the treatment of lambs with this infestation, farms use an arsenal of specific products offered by the Ukrainian market of veterinary preparations. However, their use does not always give the desired therapeutic effect. Specialists of the veterinary service most often use antiparasitic preparations, which, combined with therapeutic properties, show immunostimulating efficacy on the animal body [7]. Therefore, despite the scientific results already obtained, eimeriosis of lambs still does not lose its relevance today. Considering the current situation, it is advisable to continue scientific research on the creation of new, more effective therapeutic preparations and remedies, as well as to modify the methods of their use. Specifically, this refers to granulation of decoquinate to feed for uniform application of the preparation to animals.

The purpose of the study lied in evaluating the effectiveness of decoquinate in granulated feed in the treatment and prevention of eimeriosis in lambs and ensuring an increase in daily body weight gain.

Literature Review

Eimeriosis refers to protozoal diseases of lambs and young animals of other animal species, which is manifested by damage to the digestive tract. The disease is marked by a considerable death of animals, as well as exhaustion and a decrease in immunity. Thus, in some farms, the mortality rate of lambs was recorded within 90-100% [6]. At the same time, lambs with eimeriosis have growth retardation and developmental depression. Therewith, the quality of products is substantially reduced, which leads to economic losses in sheep breeding [6].

According to F.A. Akande and A.M. Philip (2020) [7], the invasiveness of eimeria substantially depends on the way lambs are kept in farms. Thus, in case of group keeping of animals, when they are actively in contact with each other from the first day of life, the invasion intensiveness of Eimeria increases by Days 14-21 or subsequently – by Days 25-35. After Days 45-60, the pathological process in lambs acquires maximum indicators and then the disease proceeds chronically [7; 8].

B. Bangoura and K.D. Bardsley (2020) often found Eimeria together with other single-celled pathogens and helminths of various species [9]. According to S.T. Keeton and C.B. Navarre (2018), infestation of young sheep with Eimeria shows seasonal dependence. Warm and excessively humid spring is a particularly dangerous period for lambs' infection by these pathogens. The explanation of the established pattern is that high indicators of the average monthly temperature and humidity of atmospheric air create favourable conditions for intensive development, maturation, and preservation of exogenous forms of Eimeria in the environment [10].

The seasonal dynamics of the incidence of eimeriosis in lambs is especially controlled in farms where young sheep are kept in untreated or so-called "primitive" premises. As a rule [11-13], in the conditions of industrial sheep farming, sanitary and hygienic parameters are observed in all types of livestock complexes, which has less influence on the course of eimeriosis according to the results of other authors [14]. In this case, the course of eimeriosis in these animals and its spread are regulated only by their rearing system [15].

Infected lambs release Eimeria oocysts with faeces into the environment 5-7 days after infection. Subsequently, this is recorded during the entire period of the disease [16; 17]. Eimeria is diagnosed by detecting *Eimeria* oocysts in fresh faeces using faecal flotation techniques similar to those used to establish roundworm eggs. According to H. Hoste *et al*. (2022), diagnosis should be established on a group basis, since the number of oocysts can vary substantially between individual lambs in the group [18]. In this regard, M. Acharya *et al*. (2020) [19] developed and recommended the use of eimeriostatics in animals.

Currently, manufacturers have developed a powerful arsenal of medicines to eliminate eimeriosis in sheep. Thus, H. Legendre *et al*. (2018) [20] believe that some preparations can be added to feed, water, or used as a balanced and pressed compound feed. According to A.M. López *et al*. (2019) [21], the corresponding medicinal products affect the animal body continuously or periodically. At the same time, the use of such preparations causes a decrease in the natural resistance of not only pathogens, but also the defences of the animal body.

Considering the scientific data accumulated in the world, lamb eimeriosis stays an urgent issue for applied veterinary medicine, since the species composition of pathogens has not yet been sufficiently clarified and the area of their distribution has not been definitively determined. Furthermore, morphofunctional changes in the body of lambs with eimeriosis are still understudied.

Materials and Methods

Experimental studies on eimeriosis in lambs were conducted during 2019-2022 at the scientific laboratory of the Department of Pharmacology, Parasitology, and Tropical Veterinary Medicine of the Faculty of Veterinary Medicine of the National University of Life and Environmental Sciences of Ukraine. For this purpose, groups of lambs of the Romanov breed were formed from one month to six months of age with clinical symptoms characteristic of eimeriosis – lack of appetite, diarrhoea, and unsteady gait.

Production experiments were conducted in sheep farms of the Cherkasy region, specifically two farms ("Ararat" and "Lobodiuk") and two homesteads ("ALEKS Agro "and "Rudenkovo"). These farms specialize in the production

of meat, wool, leather with wool, as well as in the production of milk. They are characterized by almost identical conditions for keeping and feeding sheep. Thus, the farms "Lobodiuk" and "Ararat" use free grazing of animals on their own haymakers, which are located on the territory of Cherkasy region, and the farms "Rudenkovo" and "ALEKS Agro" raise sheep indoors.

One experimental and one control group of 10 lambs each (n = 10) were formed to evaluate the effectiveness of decoquinate added to the granulated feed fed to lambs for the treatment and prevention of eimeriosis and to increase their daily body weight gain. All lambs had free access to ewes, pelleted feed and water.

Lambs of the experimental group were given decoquinate at 1 mg/kg in free access. The active substance was added to the over-granulated AVA ZDOROVA feed (starter granulated compound feed for lambs aged 0 to 3 months).

Individual samples of faeces from the rectum were collected for coproscopic studies in lambs. In counting the number of Eimeria oocysts in 1 g of faeces, the study adhered to the method described in DSTU 5079:2008 "Veterinary Medicine. Methods of Laboratory Diagnosis of Eimeria Diseases" [22] using a Bresser Biolux LCD Touch microscope (30x-1200x).

The species affiliation of Eimeria was established by cultivation in a 2% solution of potassium dichromate at 30°C for seven days.

The efficacy of decoquinate in sheep with eimeriosis was established by calculating the extensefficiency according to formula (1):

$$EE = 100\,\frac{E2 \cdot C1}{E1 \cdot C2} \cdot 100 \quad (1)$$

where *EE* is the extensefficiency, %; *E1* is the invasion extensiveness of experimental animals before treatment; *E2* is the invasion extensiveness of experimental animals after treatment; *C1* is the invasion extensiveness of control animals before treatment of experimental animals; *C2* is the invasion extensiveness of control animals after treatment of experimental animals.

In this case, the intensefficiency (IE) was calculated according to the formula (2):

$$IE = \frac{100 - (IIE \times II0c)}{IIc} \quad (2)$$

where: *IE* is the number of dead parasites in the experiment after disinfection, %; *IIE* is the invasion intensiveness in the experiment, %; *II0c* – initial level of II in control, eggs, oocysts/g; *IIc* – II in control, eggs, oocysts/g.

The obtained numerical results were processed statistically using SAS software version 9.X for Mac (SAS Institute Inc., USA), where the Kruskal-Wallis test was used to evaluate differences between three or more samples under experimental conditions involving ordinal data with independent measurement plans. This test regulates one-way analysis of variance. Variance analysis requires numerical scores that can be used to calculate average values. To use the Kruskal-Wallis test, it is sufficient that the data is ranked according to the change in the number of measurements. The Kruskal-Wallis test is also a mirror image of the Mann-Whitney U test. However, the Mann-Whitney U test restricts comparison to only two samples, whereas the Kruskal-Wallis test is used to compare more than three samples [23].

In addition, the intensity of invasion (II) was calculated for eimeria in lambs using a McMaster chamber (Great Britain, Marienfeld Superior).

The invasion extensiveness (IE) was calculated using the generally accepted formula (3):

$$IE = \frac{\chi}{\gamma} \text{x } 100 \quad (3)$$

where *IE* is the invasion extensiveness; χ is the number of infected animals in the group; γ is the total number of animals in the group.

To find the average daily gain of sheep during the fattening period (4) and at the age of reaching a body weight of 80 kg (5), the following formulas were used:

$$X = \frac{T2-T1}{P2-P1} \text{x} 1000 \quad (4)$$

where *X* is the average daily growth, g; T_1 is the weight of animals at the beginning of the accounting period, kg; T_2 is the weight of animals at the end of the accounting period, kg; P_1 is the age of animals at the beginning of the accounting period, days; P_2 is the age of animals at the end of the accounting period, days;

1000 is the conversion factor to grams.

$$X = B + \frac{80-m}{P} \quad (5)$$

where *X* is the age of the animal upon reaching a body weight of 80 kg, days; *B* is the real age of animals on the day of the last weighing, days; *m* is the real body weight of animals on the day of the last weigh-in, kg; *P* is the average daily growth of animals for the accounting period, kg.

The number of feed days is found by multiplying the average livestock for the reporting period by the number of days in this period [24].

Experiments involving lambs followed the "General Ethical Principles of Animal Experiments", which were approved at the National Bioethics Congress held in Kyiv in 2001 [25] and acted per the provisions of the "European Convention for the Protection of Vertebrate Animals" [25].

Results and Discussion

According to the results of research in farms and homesteads of the Cherkasy region, infection of sheep with eimeria was established. During the entire experiment, in the conditions of private and homestead farms, the intensity of infestation in lambs with eimeria increased from a minimum of 13.5% during the first month to a maximum of 95.9% in the second month of the study. According to the results of the survey of 140 animals, eimeriosis was registered in 45 lambs from farms and in 34 animals from household farms.

On average, the intensity of infestation in sheep in the herd increased by 23.9% every 14 days, which indicated the statistical need for difference (Kruskel-Wallis test; $P < 0.05$) between the following periods of the study. The exception was the period between Days 42 and 58, when the smallest increase in the invasion extensiveness was detected – by 13.2%.

According to the obtained research results, during the analysis of the species composition, representatives of the

genus *Eimeria* were recorded in most cases in lambs, namely *E. arloingi* (invasion extensiveness – 41%) and *E. crandallis* (invasion extensiveness – 29%), which dominated in the youngest animals, while less pathogenic species appeared in older animals – *E. intricate* (invasion extensiveness – 15%) and *E. faurei* (invasion extensiveness – 15%) (Fig. 1).

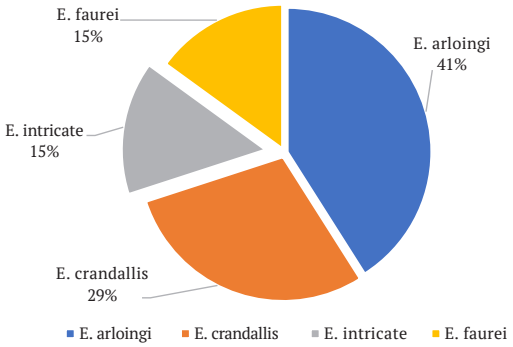

Figure 1. Infection of lambs by various types of Eimeria in farms of Cherkasy region

The research results confirm the data of other authors [5; 12; 13], where *E. arloingi* and *E. crandallis* were detected in the first period of life of lambs (Day 28). At the same time, according to the data of the literature [4], the spread of *E. intricate* and *E. faurei* was not previously recorded in lambs on the territory of Ukraine.

As is known [6], the species composition of the causative agents of eimeriosis primarily depends on the locality, more precisely, the environmental conditions, the susceptibility, and sensitivity of animals, as well as the conditions of their breeding.

Several types of Eimeria oocysts were detected simultaneously in the samples taken from the faeces, which had a detrimental effect on the general condition of the lambs' body. Thus, during a clinical study in lambs, the following was recorded:

– disorders of the gastrointestinal tract;

– dyspepsia with significant excretion of faeces with mucus and blood;

– an increase in body temperature in individual lambs to 41.5°C with a pronounced deterioration in the general condition of the body.

In individual adult sheep who reached one to two years of age, a subclinical course of invasion was noted.

In addition to scale, another important indicator characterizing the course of eimeriosis in sheep is the invasion intensiveness, expressed by the number of oocysts released per 1 g of faeces. Analysing the results of our research and the results of other researchers [15], the period of rearing lambs until Day 28 is characterized by a relatively low intensity of invasion, which indicates a slight penetration and reproduction of eimeriosis pathogens in their body.

In studies conducted in European countries, the highest level of oocysts per 1 g of faeces was established in the second month of life of lambs [13]. According to other studies [12] conducted on the territory of the Cherkasy region, the critical moment is the period between Days 38 and 50 of life of lambs, when there is less penetration of pathogens of eimeriosis into the body. In case of rapid spread of infection of sheep on the farm, oocysts in faeces can be detected from Days 21 to 33 of their life.

The highest values of the invasion intensiveness and extensiveness were recorded in two-month-old lambs, namely: IE was 100%, and the average II was 12,000 oocysts in 1 g of faeces. Lambs older than 5-6 months of age were affected less (IE – 77%, average II – 3,564 oocysts in 1 g of faeces).

The dynamics of II changes in eimeriosis in the lambs under study from the first to the second month of life is presented in Fig. 2. The average II in the study period was 7,970.2 oocysts in 1 g of faeces.

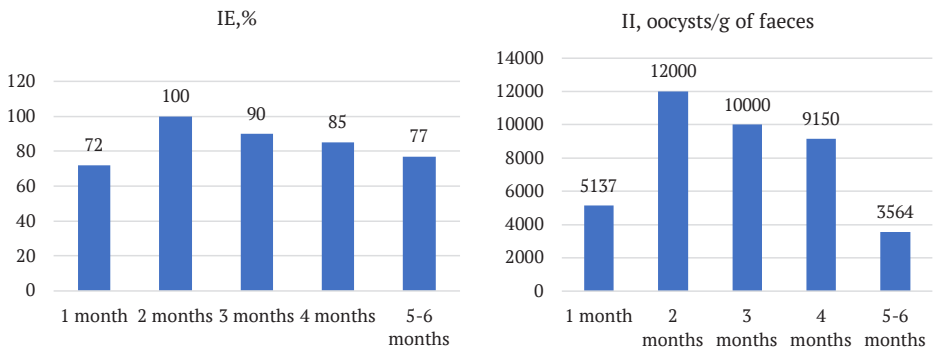

Figure 2. Dynamics of lamb infestation depending on age in farms of Cherkasy region

Before starting the experiment, the lambs of both groups were examined several times for the presence of Eimeria oocysts. It was found that all lambs were infected with Eimeria oocysts (Fig. 3). The invasion intensiveness was 10-20 oocysts in one field of view of the microscope.

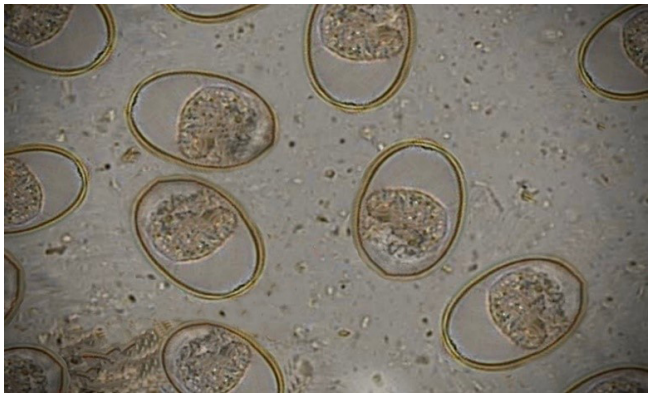

Figure 3. Eimeria oocyst of E. Arloingi in sheep (× 700)

On Day 7 of examination of faeces in the experimental group, it was established that all lambs were infested with Eimeria oocysts (Table 1). On Day 14 of the experiment, no Eimeria oocysts were detected in the faeces of five lambs, i.e., the *EE* was 50%. On Day 21 of the experiment, 8 lambs with an extensefficiency index of 80% were released from Eimeria. On Day 28, only 9 lambs were released from Eimeria infestation. Notably, one lamb remained affected by Eimeria oocysts. During the study, 2-3 oocysts were detected in the field of view of the microscope, which confirms the parasite carrier of eimeriosis pathogens.

Table 1. Indicators of invasion of lambs during treatment with decoquinate (m ± m, n = 10)

Group	Lambs released from Eimeria oocysts	EE, %	Lambs released from Eimeria oocysts	EE, %	Lambs released from Eimeria oocysts	EE, %	Lambs released from Eimeria oocysts	EE, %
	Day 7		Day 14		Day 21		Day 28	
Control	0	0	0	0	0	0	0	0
Experimental	0	0	5	50	8	80	9	90

In the control group of lambs, both at the beginning of the experiment and on Day 28 of the study, all 10 animals stayed infected with Eimeria. Therewith, the invasion intensiveness even slightly increased from 10-20 to 15-29 oocysts in the field of view of the microscope (Fig. 4). Lambs of the experimental and control groups were weighed 1-2 hours after morning feeding. The results of average control weighings are presented in Table 2.

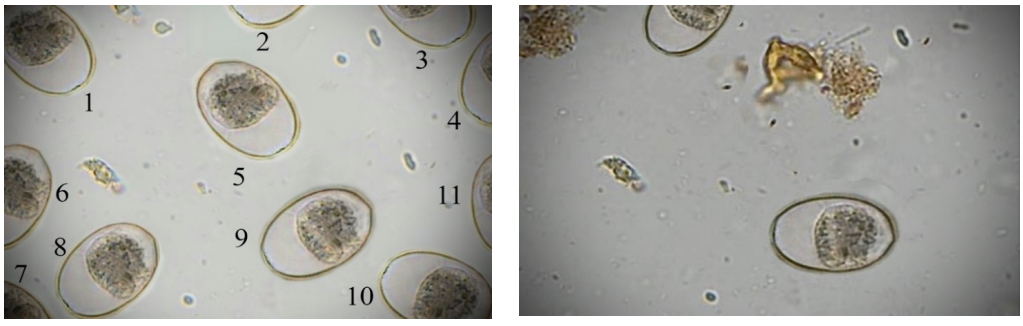

Figure 4. Photo of oocysts in the same field of view of the microscope in the control and experimental group on Day 28 of the experiment; numbers indicate Eimeria oocysts of E. Arloingi in sheep (× 700)

Table 2. Dynamics of lamb body weight gain (m ± m, n = 10, kg)

Group	Before the study	Day 7	Day 14	Day 21	Day 28
Control	9.45±0.18	10.47±0.17	11.40±0.20	12.66±0.20	13.80±0.21
Experimental	9.40±0.18	10.40±0.17	11.72±0.20	13.20±0.20**	14.60±0.21*

Note: * P < 0.05, ** P < 0.01, compared to the indicators of the control group

According to the results obtained, the absence of relative weight gain of lambs on Days 7 and 14 of the experiment was established. On the other hand, 21 days after the start of the study, the body weight of lambs increased by 4.27% ($P < 0.01$), and on Day 28 – by 5.80% ($P < 0.05$) compared to Day 21 of the study.

The average daily gain was 155.36 g in the control group and 185.71 g in the experimental group, which confirms the effectiveness of decoquinate fed to lambs.

In various farms in the Cherkasy region, infection with Eimeria of lambs was noted, regardless of age group. The maximum values of their Eimeria invasion were detected at the age of two months. Furthermore, there is a pattern where indicators of the invasion intensiveness and extensiveness of lambs decrease with age.

Therewith, the positive effectiveness of decoquinate was found, provided that it is used at a dose of 1 mg/kg of animal weight for 28 days. This provided an extensefficiency of 90%. In 10% of lambs, the II did not exceed 2-3 oocysts in the field of view of the microscope, and they stayed parasitic carriers of eimeriosis pathogens. Importantly, the use of decoquinate by adding it to the pre-granulated feed AVA ZDOROVA (AVA GROUP Ukraine) at 1 mg/kg for eimeriosis leads to an increase in average daily growth by 19.6%.

Thus, with eimeriosis of lambs after the period of the highest II, its significant decrease is noted. The number of oocysts released with lamb faeces, as a rule, is steadily decreasing. This dependence is confirmed by the results of the conducted studies. As demonstrated by statistical analysis, the increase in mean value observed in the second month of the study was the result of high values recorded in individual lambs. It should be emphasized that adult sheep have a constant but low level of infection with Eimeria oocysts from the environment. Thus, adult sheep become asymptomatic parasitic carriers of eimeriosis pathogens and, at the same time, a reservoir and source of infection of lambs in farms of various forms of management and, especially, with their intensive cultivation.

Data from the literature [19] and the results of our research indicate that in lambs kept in farms with an intensive breeding system, the degree of infection with eimeria pathogens can reach more than 96%. With low II, an asymptomatic course of the disease is possible; therewith, animals stay infected with Eimeria. Therefore, it is important to carry out certain preventive measures with such sheep. Otherwise, disregarding such measures may lead to significant economic losses in the farm.

It is noted that II increases between the first and second months of lambs' life, after which there is a period of gradual and constant release of Eimeria oocysts with faeces into the environment. However, the number of Eimeria oocysts can vary significantly depending on the general condition of the lambs. When conducting microscopic studies, oocysts of one type of Eimeria or more of them compared to others were detected more often. However, oocysts of other Eimeria species are somewhat less common.

At the same time, the factor that accelerates both the spread of eimeriosis and II is the early weaning of lambs from ewes, as well as untimely preventive measures, and, if necessary, preventive treatment. In this regard, the best solution to the prevention of eimeriosis is keeping lambs together with ewes as long as possible, as well as the use of chemotherapeutic agents that prevent the occurrence of invasion and contribute to the improvement of their body's defences, improve the quality of life and stimulate productivity.

Conclusions

According to the data obtained, in farms of the Cherkasy region, the prevalence of eimeriosis in lambs during the year ranges from 72-100%, and in household farms – reaches 90-100%. In all types of farms, this protozoan disease is caused by pathogens: *E. arloingi* (IE – 41%), *E. crandallis* (IE – 29%), *E. faurei* (IE – 15%), and *E. intricate* (IE – 15%). The highest values of the investigated indicators are typical for two-month-old lambs, namely: IE – 100% and average II – 12,000 oocysts in 1 g of faeces.

The efficacious influence of decoquinate is noted at 1 mg/kg body weight of lambs when used for 28 days. The extensefficiency is 90%. 10% of lambs stay parasitic carriers of eimeriosis pathogens. The invasion intensiveness in them is 2-3 oocysts in the field of view of the microscope.

The therapeutic effect of decoquinate at 1 mg/kg, added to AVA ZDOROVA feed (starter granular compound feed, produced by AVA GROUP Ukraine), is manifested in lambs already on Day 14 after feeding; the extensefficiency is 50%, and on Day 28 – 90%.

The average daily gain is 155.3 g in the control group, and 185.7 g in the experimental group, which in turn confirms the effectiveness of the decoquinate used in lambs.

Thus, for effective treatment of sheep with eimeriosis, the authors of this study recommend using decoquinate with granulated compound feed produced by AVA GROUP (Ukraine).

In the future, it is planned to find the features of the pathogenesis and spread of eimeriosis in lambs in other regions and to analyse the effectiveness of the available preventive measures; to recommend new developments for use; to systematize and propose the organization of scientifically based medical and preventive measures in sheep farms.

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Ефективність декоквінату в гранульованому кормі за еймеріозу ягнят

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Анотація. За еймеріозу овець знижується рентабельність виробництва вовни, шкіри, м'яса та молока, що завдає істотних збитків галузі вівчарству, а тому потребує впровадження на виробництві ефективних засобів профілактики та лікування тварин за цієї інвазії. Метою дослідження було визначити ефективність декоквінату в гранульованому кормі за еймеріозу ягнят. У результаті проведення копроскопічних досліджень згідно з ДСТУ 5079:2008 «Ветеринарна медицина. Методи лабораторної діагностики еймеріозів» визначено екстенсивність інвазії ягнят ооцистами різних видів еймерій, зокрема, *E. arloingi* – 41 %, *E. crandallis* – 29 %, *E. intricate* – 15 %, *E. faurei* – 15 %, які реєструються у них в окремих господарствах Черкаської області. Клінічні симптоми еймеріозу спостерігаються у молодняка з одномісячного віку і характеризуються анемічністю видимих слизових оболонок, діареєю, пригніченням загального стану, слабкістю та переважно гострим перебігом інвазії з високою летальністю. Прояв клінічних симптомів хвороби залежить від багатьох факторів, зокрема, віку ягнят, санітарної ситуації в господарстві, зміни кормових програм відгодівлі, стресових ситуацій, що виникають внаслідок переміщення овець в інші приміщення або відлучення ягнят від вівцематок. У ягнят старшого віку, як правило, хвороба має хронічний та безсимптомний перебіг. У таких тварин оцисти еймерій виділяються з фекаліями у значно меншій кількості, ніж в одномісячних ягнят за гострого перебігу інвазії. Максимальні значення екстенсивності інвазії (100 %) та інтенсивності інвазії (12000 ооцист в 1 г фекалій) встановлено в двомісячних ягнят. За інвазії у травному каналі цих тварин погіршується засвоєння поживних речовин корму, що сприяє зменшенню середньодобового приросту маси тіла (в середньому 155,3 г/добу), який зростає за лікування тварин декоквінатом (в середньому 185,7 г/добу). Загалом, застосування хворим ягнятам препарату декоквінату в дозі 1 мг/кг маси тіла, впродовж 28 діб виявляє високу лікувально-профілактичну ефективність. При цьому, екстенсефективність становить 90 %, а паразитоносійство відмічається лише в 10 % тварин. Тому, для ефективного лікування ягнят різних вікових груп може бути рекомендований препарат декоквінат, що особливо актуально для господарств з інтенсивним вирощуванням молодняку овець

Ключові слова: ооцисти, Eimeria, інвазія, лікування, методи діагностики

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Prospect of Using *B. Anthracis* Exotoxin in the Design of Anti-Selective Emergency Preparations

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Abstract. The relevance of the study is conditioned upon the fact that outbreaks of anthrax are periodically recorded on the territory of Ukraine, not only in ruminants, but also in pigs, fur animals, dogs, and people. The purpose of the study is to investigate the protective properties of the experimental vaccines and the abacillary vaccine "Antracol" and to prove the immunogenic effect of the extracellular toxin from the *B. anthracis* K-79 Z strain. Cultures of vaccine strains of anthrax were used for the experiments: *B. anthracis* 55, *B. anthracis* SB, *B. anthracis* K-79 Z and the "Antracol" vaccine (experimental development). Microbiological, clinical-biological, and biotechnological research methods were used in the study. The protective effect was investigated on guinea pigs (*Cavia porcellus*). An acute experiment was performed with a virulent strain *B. anthracis* 92 Z. Exotoxin was obtained from the specified cultures. The titre of the exotoxin was found in the disk precipitation reaction. The highest result regarding exotoxin production was recorded in *B. anthracis* K-79 Z 1 : 128 with a total protein concentration of 0.19 mg/ml, while the exotoxin of *B. anthracis* strain 55 with a titre of 1 : 32 showed a high total protein concentration of 0.4 mg/ml. The effect of *B. anthracis* exotoxins on the body was investigated by administering them to laboratory animals in different titres of exotoxins, followed by infection with the pathogenic strain *B. anthracis* 92 Z. The exotoxin of the vaccine strain *B. anthracis* K-79 Z in a titre of 1 : 64-1 : 128 shows the best protective properties against the pathogenic strain. It was found that the vaccine strains of *B. anthracis* SB and *B. anthracis* K-79 Z have the same level of protection of laboratory animals during experimental infection, which is 60%, while the vaccine from the strain *B. anthracis* $34F_2$ showed a level of protection of 20%. Based on the results of the study, it was found appropriate to use exotoxin *B. anthracis* in the development of prophylactic preparations against anthrax. The research results can be used by scientists and specialists in the field of veterinary medicine to develop new and improve the available vaccines for effective anthrax prevention

Keywords: anthrax, metabolites, preventive effect, abacillary vaccine "Antracol"

Introduction

The main problem of the study is anthrax, a zoonotic disease that affects humans, domestic, and wild animals. First of all, this is a disease of herbivores [1]. In economically developed countries, it is rare, thanks to the developed system of total vaccination of susceptible livestock and prompt control of anthrax burial sites. Anthrax is a disease registered on all continents, as the infection is of natural origin and occurs in the farming communities of

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developing countries. Anthrax is still an endemic disease in some parts of the world, namely in the countries of Central Asia, in many regions of the Russian Federation, Africa (south of the Sahara Desert), Central and South America, especially in arid and tropical rural areas [2; 3]. According to statistics, the incidence of anthrax worldwide ranges from 20,000 to 100,000 cases per year. Therewith, about 63.8 million pastoralists and 1.1 billion pets are at risk [4].

The scientific literature [5-7] contains a lot of information about anthrax research. Presently, a critical area is the development and improvement of existing avirulent spore vaccines, as well as the study of reactogenicity of preparations and treatment protocols. Special attention is paid to studying the mechanism of action of anthrax exotoxins and their effect on the immune system of animals [6]. The spore formation of anthrax strains and the mechanism of action at the molecular and genetic level of composite vaccines during the development of infection are investigated [7]. Currently, the issue of creating a combined preparation against anthrax as a preventive and curative agent that can be used in foci of infection outbreaks stays unresolved [8].

Currently, only live spore anthrax vaccines are registered and used in Ukraine. They have many limitations: they are used for ruminants aged from 8 months and a satisfactory immune state. The production of such prophylactic preparations is also conditioned upon the risk to the health of people who work with the pathogen (anthrax belongs to microorganisms of the pathogenicity group II, it can lead to infection of service personnel) [9]. The abacillary vaccine will solve the problem of vaccination of all mammals. The design of such a preparation is safe for the environment and humans. Researchers of the "State Centre for Innovative Biotechnologies" conducted several experiments that yielded positive results on the creation and use of vaccines that contained the products of the anthrax pathogen's vital activity in the absence of spores of the bacillus itself [10-12].

Studies of spore-forming bacteria have shown their resistance to chemicals and elevated temperatures. This property can vary both in diverse types of microorganisms and in strains of the same species [13]. It was established that variable sporulation conditions, including the use of liquid or solid media, levels of nutrients and divalent cations, as well as pH and temperature of the sporulation medium, can affect the persistence properties of the resulting spores [14].

Contamination of the environment with virulent pathogens requires the creation of protection for animals and people in the shortest possible time. Modern vaccines do not meet these requirements because immunity is formed much longer (14-30 days) than the incubation period of the disease. Considering the danger of zooanthroponotic infection, there is a need for a quick and effective response to anthrax outbreaks. Thus, the creation of new and improvement of available means of anthrax prevention is a relevant and urgent need.

In the 20th century, anthrax outbreaks were recorded in most regions of Ukraine, which led to a tense epizootic situation. Before the development and introduction into production of the first Ukrainian vaccine "Live vaccine against animal anthrax from the K-79Z strain" (author academician A.I. Zaviriukha), outbreaks of anthrax were registered annually, mainly among cattle (71.7%) [15]. Thus, in 1999-2009, the largest number of outbreaks (53.8-75%) was observed in the Chernivtsi, Vinnytsia, Kharkiv, Cherkasy, Luhansk, Mykolaiv, Rivne, Donetsk, Kirovohrad, and Kyiv regions of Ukraine [15]. In addition, animals infected with anthrax and the products of their forced slaughter infected and sickened people – 30 cases of the disease over 20 years in 9 regions of the country [15]. Outbreaks were registered mainly due to insufficient intensity and duration of specific immunity formed after vaccination with the vaccine "Live vaccine against anthrax from the STI strain" of Agrovet LLC, which was used until 1998 [9]. The intensive spread of anthrax on the territory of Ukraine was also facilitated by black earth soils, which are a suitable environment for the presence of *B. anthracis* spores [1; 9; 15].

The basis for the prevention and control of anthrax is vaccination of animals. Modern commercial anthrax vaccines are made from spores of vaccine strains. A distinctive feature of the biology of the anthrax pathogen is the production of extracellular toxin [16; 17].

The use of bacterial exotoxins has become an innovative approach in the treatment and prevention of many diseases of viral and bacterial aetiology. In Ukraine, spore vaccines from strains Sterne, K-79 Z, UA-07, SB are used for preventive and forced vaccinations of animals against anthrax. Despite the effectiveness of modern anti-selective vaccines, they need to be improved. An important criterion is an increase in the duration of immunity, an increase in the production of protective antigen by vaccine producing strains.

The purpose of this study is to investigate the protective properties of the experimental vaccines and the abacillary vaccine "Antracol" and to prove the immunogenic effect of the extracellular toxin from the *B. anthracis* K-79 Z strain, which is part of this vaccine preparation.

Literature Review

The main means of preventing and controlling anthrax is vaccination of animals, and in case of infection – use of antibiotics. The most effective against *B. anthracis* are penicillin, amoxicillin, levofloxacin, and ciprofloxacin. It is proved that anthrax is a toxigenic disease, in which the cessation of bacterial growth in the host/carrier body under the action of antibiotics does not mean a positive dynamic of the disease. If the toxins enter the host/carrier cell in sufficient quantities, they can show their pathogenic effects for up to 5 days [18-20]. Anthrax spores persist in soil or water for hundreds of years. Anthrax spores can withstand direct sunlight for 10 days. They can also be stored for years in animal corpses, wool, and salted meat. Anthrax spores germinate after entering the body through damaged areas of the skin (cutaneous anthrax) either by air (inhaled anthrax) or with food (gastrointestinal form of anthrax). Most spores germinate within 48 hours, but this pathway of pathogen development can occur up to 60 days. While the inhaled form of anthrax almost always results in a lethal outcome, the intestinal form is fatal in 25-60% of cases. Up to 20% of patients with the cutaneous form of anthrax die, and the remaining animals recover [21].

In Ukraine, spore vaccines from strains Sterne, K-79 Z, UA-07, SB are used for preventive and forced vaccinations of animals against anthrax [22; 23]. A distinctive

feature of the biology of the anthrax pathogen (field and vaccine strains) is the production of an extracellular toxin. This product of the metabolism of the vegetative form of bacilli consists of three fractions: a) protective, b) edematous, and c) lethal. Both field and vaccine strains of the anthrax pathogen can produce a toxin. *B. anthracis* has two major virulence factors: a poly-γ-D-glutamic acid capsule and a tripartite toxin [23-25]. The capsule plays an antiphagocytic role, which allows bacteria to avoid absorption by macrophages. The tripartite toxin includes protective antigen (PA), lethal factor (LF) and edema factor (EF) [26]. These three proteins combine to form two toxins, a lethal toxin (LT, a combination of PA and LF) and an edematous toxin (ET, a combination of PA and EF). Toxins alter the signalling pathways of cells in a living organism to interfere with the innate immune response in the initial stages of infection and cause vascular collapse in the later stages of the disease. Toxins affect the innate and adaptive immune systems and the subsequent effects of the disease. Numerous studies prove that the virulence of the anthrax pathogen, which has lost the ability to form a capsule, decreases, while its immunogenic properties are preserved [27; 28]. Despite the effectiveness of modern vaccines, scientists are searching for the development of the most areactogenic preparations, namely the improvement of anthrax vaccines to reduce irritation and inflammation at the injection site. Similar reactions occur in case of an edematous and lethal factor [29-31]. Another important criterion is an increase in the duration of immunity, an increase in the production of protective antigen by vaccine producing strains. Such opportunities are provided by genetic engineering methods, since manipulation with the genes of plasmids pXO1 and pXO2 allows increasing the immunogenicity of anthrax vaccine strains by introducing added, foreign genetic fragments [32; 33]. Modern commercial anthrax vaccines are made from spores of the vaccine strains and are a spore suspension preserved in 30% glycerol with or without the addition of appropriate adjuvants. The reliability of such vaccines and the prolongation of post-vaccination immunity primarily depends on the immunobiological properties of the anthrax strain used for vaccine production. Almost all anthrax vaccines in use and under development are based on the production of the exotoxin PA as the main immunogen. The BioThrax vaccine in the US (formerly known as AVA) and the similar AVP vaccine in the UK are alum-adsorbed culture filtrates of vaccine strains of the anthrax pathogen that contain PA. Earlier studies have shown that exotoxin immunization of various animal models provides protection against anthrax infection [29-31]. A post-vaccination correlation of PA antibody titres has also been established [32; 33]. In new generations of vaccines, improvements are aimed at reducing toxicity, allergenic action, methods, and doses of administration and compliance with modern requirements for the quality of immunobiological preparations [34-36].

The creation of vaccines against anthrax based on mutant variants of PA exotoxin, which have lower toxicity and greater immunogenicity, is a promising area in the development and improvement of vaccines. Preclinical and clinical trials of vaccine candidates are being conducted [37-39]. Protein-based subunit vaccines are a safe alternative. They are less reactogenic, are characterized by an immunogenic orientation, belong to purified bacterial preparations and, as a rule, do not cause side immunological effects during immunization. Nanolipoprotein particles (NLP) containing Toll-like receptor 4 agonist monophosphoryl lipid A (MPLA) were used as a platform for intranasal vaccination against *Bacillus anthracis*. Modified lipids made it possible to attach different antigens of spore proteins and toxins. Administration of combinations of constructs induced responses of several antigens, indicating the potential for a polyvalent vaccine. No off-target responses to the NLP framework protein were detected. Thus, the NLP platform has been proven to enhance humoral and mucosal responses to intranasal immunization. This indicates the promise of NLP as a flexible and reliable vaccine platform against *B. anthracis* [40].

Protein-based subunit vaccines are a safer alternative because they are less reactogenic, characterized by immunogenicity, belong to purified bacterial preparations and, as a rule, do not cause adverse immunological effects after immunization. Such preparations show a low allergenic effect and reduced toxicity to the body of laboratory animals. The limitations of physiological instability and low immunogenicity of such vaccines require an efficient delivery system to stimulate strong immune responses. Trials of an antigen delivery system based on the bacteriophage T4 capsid have been reported [40], with a high probability of eliciting both humoral and cellular immune responses without adjuvant. Such an anthrax vaccine, using a T4 capsid platform to display and deliver the protective antigen 83 kDa PA, a key component of the anthrax toxin, can be used as a rapid response preparation [40].

The US-licensed anthrax vaccine adsorbed (AVA) and UK anthrax vaccine precipitated (AVP) can generate a protective immune response but are associated with side effects. This refers to the reactogenicity of vaccines and the frequency of administration, age restrictions. Therefore, work on the investigation and improvement of preparations continues [41].

Production of anthrax vaccine (AVP) in the United Kingdom has focused on the production of PA from the *Bacillus anthracis* Sterne strain. Although it has been used for decades, some fundamental properties of AVP are understudied, including its exact composition and which proteins other than PA may contribute to protection. Recent studies have shown [42-44] that the effectiveness of AVP in humans may depend not only on PA. Firstly, there is strong evidence that LF plays a protective role, and computational predictions suggest that added proteins may be important in individuals with specific combinations of HLA alleles [43; 45; 46]. Secondly, despite the differences in the sequences of key antigenic proteins in different strains of *B. anthracis*, it is unlikely that they will affect the cross-strain protection provided by AVP [47-49].

Currently, scientific studies of prophylactic preparations against anthrax AV 7909 are being conducted as next-generation vaccines for post-exposure prophylaxis (PEP) [26]. AV 7909 consists of bulk drug substance adsorbed to anthrax vaccine (AVA, BioThrax®) with an adjuvant immunostimulatory oligodeoxynucleotide (ODN) compound, CPG 7909. The addition of CPG 7909 makes AV 7909 an acceptable next-generation vaccine for use in post-exposure prophylaxis. Immunization with AV 7909 induced a rapid protective response of toxin-neutralizing antibodies

in guinea pigs. The toxin-neutralizing antibody threshold associated with a 70% chance of survival for AV 7909 immunized animals was found to be significantly lower than that established for the licensed AVA vaccine [26; 35].

The use of bacterial exotoxins has become an innovative approach in the treatment and prevention of many diseases of viral and bacterial aetiology. Experimental preparations containing exotoxins demonstrate *in vivo* rapid growth of macrophages, and *in vitro* – high preventive activity and formation of immunity in laboratory animals. It has been proven that PA is a harmless subunit of the toxin that causes a protective immune response and constitutes the basis for all preventive measures against anthrax. Accordingly, anthrax protein vaccines licensed for human use include partially purified PA fortified with various adjuvants [44; 45].

Materials and Methods

The study was conducted at the State Scientific Institution (SSI) "State Center for Innovative Biotechnologies" during 2018-2021 under state budget topics: No. 0111U0006386 "Scientific research to develop methods of early prevention and treatment of infectious diseases common to humans and animals" and No. 0119U100900 "Exotoxin of the causative agent of anthrax. Characterization, identity between strains, an alternative to antibiotic therapy".

The extracellular toxins of *B. anthracis* have become the basis for new experimental preparations, which are an alternative to the use of antibiotics in case of anthrax outbreaks and are not limited in applications.

The following vaccines against anthrax were used: *B. anthracis* 55 (FRCVM), *B. anthracis* SB (Sumy biofactory, Ukraine), *B. anthracis* K-79 Z (Ukraine, Kherson biofactory) and "Antracol". An experimental series of bacterial-free (abacilar) vaccine "Antracol" was created at the State Scientific Institution "State Centre of Innovative Biotechnologies". The inventors of this preparation are A.I. Zaviriukha, H.A. Zaviriukha. The main feature of "Antracol" is that the vaccine is abacillary, contains products of cultivation of the vaccine strain and differs in that the titre of anthrax protein in the products of cultivation of this strain is 1 : 2 – 1 : 512. The component of the vaccine is the extracellular toxin – the protective antigen of *B. anthracis* K-79 Z, deposited by the State Scientific Control Institute of Biotechnology and Strains of Microorganisms (State Scientific Control Institute of Biotechnology and Strains of Microorganisms) under No. 069. There are no analogues of this vaccine in Ukraine.

Vaccine preparations were manufactured per TUU 46.15.132.96 [50]. Exotoxins of vaccine strains *B. anthracis* 55, *B. anthracis* $34F_2$, *B. anthracis* K-79 Z, virulent strain *B. anthracis* 92 Z were used.

Before conducting the experiment, the experimental cultures were tested for purity by inoculation on nutrient media: Nutrient Agar HIMEDIA, Nutrient Broth HIMEDIA, Meat-peptone broth (MPB), Meat-peptone agar (MPA).

Cultures were involved in the experiment, in which the number of microbial cells in 1 cm^3 of culture liquid was as follows: *B. anthracis* K-79 Z – 30.5×10^6 colony-forming units (CFU), for *B. anthracis* 55 – 114×10^6 CFU, *B. anthracis* SB – 20.4×10^6 CFU. The specified number of microorganisms was used to obtain exotoxins with different titres. Exotoxins of the specified strains were obtained by filtration using a bacterial candle [51]. After figuring out the concentration of total protein in the exotoxins of *B. anthracis* vaccine strains, the following studies were conducted on guinea pigs to investigate the effect of metabolites of the anthrax pathogen on their bodies when infected with the virulent *B. anthracis* 92 Z *strain.* Experimental guinea pigs were injected with exotoxins of vaccine strains in different titres. Subsequently, the animals were infected with a virulent *B. anthracis* 92 Z strain. *Cavia porcellus* guinea pigs were used as laboratory animals (n = 80, m = 324.4 ± 2.30 g) and (n = 30, m = 334 ± 2.26 g).

The animals were given exotoxins of vaccine strains in different titres. Titres were identified by the Ascoli reaction or disc reaction-precipitation (DRP). From the vaccine strains *B. anthracis* 55, *B. anthracis* $34F_2$, *B. anthracis* SB and K-79 Z, suspensions were prepared for experiments, which were checked for purity and typicality of growth, motility, and the number of microbial cells in 1 cm^3 was determined. Glycerin was added to the spore suspension to obtain a final concentration of 30%. Each vaccine contained 10 million live spores in 1 cm^3. Immunization was performed subcutaneously in a dose of 1 cm^3 near the base of the tail. The "Antracol" vaccine is abacillary and contains the extracellular toxin of *B. anthracis* K-79 Z in a titre 1 : 2 – 1 : 256, a specific anthrax protein by the disc reaction-precipitation or Ascoli.

A virulent strain of B. anthracis 92 Z (SSI National Center for Innovative Biotechnologies) was used to infect laboratory animals. To compare it with vaccines used on the territory of Ukraine, employees conducted an experiment with infection of laboratory animals. Guinea pigs were vaccinated with the above-mentioned vaccines, and then, three days later, they were infected with a pathogenic strain of anthrax.

To investigate the immunogenic effect of experimental vaccines, animals were placed in six sections, 5 animals for each vaccine preparation, including control. The study was conducted on clinically healthy laboratory guinea pigs. Before starting the study, each animal was weighed, and groups of analogues were formed. The animals were placed in separate boxes for 6 days (quarantine), provided with a balanced diet and pathogen-free drinking water. In the room where the animals were located, the temperature was at 24°C.

Animals of the control group were injected subcutaneously with a 0.9% solution of sodium chloride in the amount of 1 cm^3 around the base of the tail. A virulent strain of *B. anthracis* 92 Z (SSI National Centre for Innovative Biotechnologies) was used to infect laboratory animals. The dose of infection with this pathogen was 500,000 spores (LD_{100}). The strain was injected in the amount of 0.5 cm^3 into the abdomen 72 hours after vaccination. The animals were observed for 10 days. Experiments on animals were conducted in the vivarium of the Institute of Veterinary Medicine of the National Academy of Agrarian Sciences.

Experiments on laboratory animals were conducted according to the general principles of animal experiments, considering the requirements of the "European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Purposes" (Strasbourg, 1986) [52], "European Convention for the Protection of Domestic Animals" (2013) [53] and the Law of Ukraine "On the Protection of Animals from Cruelty" (2006) [54]. Statistical processing of experimental results was performed using the Microsoft Excel program.

Results and Discussion

According to the results of experimental studies, it was established that epizootic strains of *B. anthracis* do not produce enough exotoxin during cultivation in liquid nutrient media. Whereas the vaccine strains of anthrax produce a toxin with a different titre, which is determined by the disc reaction-precipitation.

According to the results presented in Table 1, the total protein concentration in exotoxins of vaccine strains is not the same.

Table 1. Total exotoxin protein of vaccine strains *B. anthracis* K-79 Z, *B. anthracis* SB and *B. anthracis* 55

No.	Strain	Titre for the disc reaction–precipitation	Total protein concentration, mg/ml
1	*B. anthracis* K-79Z	1 : 32	0.39
2	*B. anthracis* K-79Z	1 : 64	0.20
3	*B. anthracis* K-79Z	1 : 128	0.19
4	*B. anthracis* SB	1 : 16	0.56
5	*B. anthracis* SB	1 : 32	0.40
6	*B. anthracis* 55	1 : 16	0.50
7	Nutrient broth	–	0.57

Note: The study was conducted in compliance with the rules of asepsis and antiseptics

As Table 1 shows, a lower total protein concentration is set at a titre 1 : 32. The exotoxin of *B. anthracis* 55 strain with a titre of 1 : 16 is characterized by a high concentration of total protein. The study of exotoxin *B. anthracis* K-79 Z showed that the concentration of the total protein decreases as the titre of the specific anthrax antigen increases. Its lowest content was found in the exotoxin of the *B. anthracis* K-79 Z strain with a titre 1 : 128. This indicates the effectiveness of using this vaccine strain to produce the exotoxin with the highest titre.

Table 2 presents the results of experimental studies on the effect of anthrax pathogen metabolites on the body of guinea pigs during their infection with the virulent strain *B. anthracis* 92 Z.

Table 2. The effect of metabolites of the anthrax pathogen on the body of guinea pigs during their infection with the virulent *B. anthracis* 92 Z strain (n = 80, m = 324.4 ± 2.30 g)

Group No.	Number of animals, n	First and second immunization, volume	Exotoxin, titre	LD infection with *B. anthracis* 92Z	Died	Survived
1	5	1 cm^3 + 1 cm^3	0.9% NaCl	LD_{100} (500×10^3 CFU)	5	–
2	5	1 cm^3 + 1 cm^3	*B. anthracis* K-79 Z, 1 : 32	LD_{100} (500×10^3 CFU)	5	–
3	5	1 cm^3 + 1 cm^3	*B. anthracis* K-79 Z, 1 : 64	LD_{100} (500×10^3 CFU)	5	–
4	5	1 cm^3 + 1 cm^3	*B. anthracis* K-79 Z, 1 : 128	LD_{50} (250×10^3 CFU)	–	5
5	5	1 cm^3 + 1 cm^3	0.9% NaCl	LD_{50} (250×10^3 CFU); 2 heads infected with LD_{25} (125×10^3 CFU)	5	–
6	5	1 cm^3 + 1 cm^3	*B. anthracis* K-79 Z, 1 : 32	LD_{50} (250×10^3 CFU);	5	–
7	5	1 cm^3 + 1 cm^3	*B. anthracis* K-79 Z, 1 : 64	LD_{50} (250×10^3CFU);	–	5
8	5	1 cm^3 + 1 cm^3	*B. anthracis* K-79 Z, 1 : 128	LD_{50} (250×10^3 CFU);	–	5
9	5	1 cm^3 + 1 cm^3	*B. anthracis* 55, 1 : 16	LD_{50} (250×10^3 CFU);	5	–
10	5	1 cm^3 + 1 cm^3	*B. anthracis* $34F_2$, 1 : 16	LD_{50} (250×10^3 CFU);	5	–
11	5	1 cm^3 + 1 cm^3	*B. anthracis* $34F_2$, 1 : 16	LD_{100} (500×10^3 CFU)	5	–
12	5	1 cm^3 + 1 cm^3	*B. anthracis* 55, 1 : 16	LD_{100} (500×10^3CFU)	5	–

Table 2, Continued

Group No.	Number of animals, n	First and second immunization, volume	Exotoxin, titre	LD infection with *B. anthracis* 92Z	Died	Survived
13	5	1 cm^3 + 1 cm^3	*B. anthracis* K-79 Z, 1 : 128	LD_{25} (125×10^3 CFU)	–	5
14	5	1 cm^3 + 1 cm^3	*B. anthracis* K-79 Z, 1 : 64	LD_{25} (125×10^3 CFU)	–	5
15	5	1 cm^3 + 1 cm^3	*B. anthracis* K-79 Z, 1 : 34	LD_{25} (125×10^3 CFU)	5	–
16	5	1 cm^3 + 1 cm^3	*B. anthracis* $34F_{2,}$ 1 : 16	LD_{25} (125×10^3 CFU)	5	–
Total	80				55	25

Note: Strains were cultured, and exotoxins were obtained in nutrient broth at 37.0°C for 48 hours

The results of experimental studies presented in Table 2 show that the exotoxin of the vaccine strain *B. anthracis* K-79 Z in a titre 1 : 64-1 : 128 has the best protective properties against the pathogenic *B. anthracis* 92 Z strain.

Vaccine strains of foreign origin (*B. anthracis* $34F_2$ and *B. anthracis* 55) do not produce exotoxins in high titres according to the Ascoli reaction, and therefore they do not have sufficient immunogenic properties. The results of experimental studies on testing anthrax vaccines for the formation of immunity in animals are presented in Table 3.

Table 3. Results of testing anthrax vaccines for the formation of immunity in animals (n = 30, m = 334±2.26 g), M ± m.

Vaccine	Day											M±m
	Number of vaccinated animals that survived infection, %											
	1	2	3	Introduction of *B. anthracis* 92 Z	4	5	6	7	8	9	10	P ⩾
B. anthracis 55	5, 100%	5, 100%	5, 100%		1, 20%	1, 20%	0	0	0	0	0	2.1 ± 1.84 ⩾ 0.99
B. anthracis $34F_2$ *Sterne*	5, 100%	5, 100%	5, 100%		4, 80%	1, 20%	1, 20%	0	0	0	0	2.1 ± 1.84 ⩾ 0.96
B. anthracis SB	5, 100%	5, 100%	5, 100%		3, 60%	3, 60%	2, 50%	2, 50%	2, 50%	2, 50%	2, 50%	3.11 ± 1.09 ⩾ 0.66
B. anthracis K-79 Z	5, 100%	5, 100%	5, 100%		3, 60%	3, 60%	3, 60%	3, 60%	3, 60%	3, 60%	3, 60%	3.6 ± 0.73 ⩾ 0.47
Anthracosis	5, 100%	5, 100%	5, 100%		5, 100%	4, 80%	3, 60%	3, 60%	3, 60%	3, 60%	3, 60%	3.6 ± 0.73 ⩾ 0.47
Control	5, 100%	5, 100%	5, 100%		3, 60%	2, 50%	1, 20%	1, 20%	0	0	0	2.2 ± 1.6

Note: 10 days before vaccination, animals should not be given antibiotics, they were kept in quarantine for 10 days before the start of the experiment

The results of experimental studies presented in Table 3 indicate that the vaccine from the *B. anthracis* 55 strain has the least immunogenic activity. Three days after infection (five days after immunization), only one guinea pig survived, i.e., 20% of the total number of animals involved in the experiment. On Day 6, all animals died.

The vaccine from the *B. anthracis* $34F_2$ Sterne strain has an average level of immunogenic activity. Three days after infection of guinea pigs with the pathogenic strain, not a single animal died, the protection was 100%.

On Day 4, 1 animal died, protection was 80%. On Days 5 and 6, one guinea pig in each group survived, which accounted for 20% of the protection, and on Day 7, all experimental animals died (Table 3).

The vaccine strains *B. anthracis* SB and *B. anthracis* K-79 Z have almost the same level of protection of laboratory animals during experimental infection. Three days after infection of guinea pigs with the pathogenic strain, not a single animal died, the protection was 100%. On Day 5, 2 animals died in each group, which accounted for 60% of the protection. Starting from Day 6 and until the end of experimental studies, not a single guinea pig died. It can be assumed that these are genetically related strains.

The abacillary vaccine "Antracol", made from the extracellular toxin of *B. anthracis* K-79 Z, protects infected guinea pigs during the first three days – 100%. On Day 10 after the introduction of the pathogenic strain, 60% of guinea pigs stayed alive in the experiment.

In the control group of animals injected with 0.9% normal saline (NaCl), 100% death of guinea pigs was recorded on Day 8 of experimental studies (Table 1).

Considering the world experience in the use of exotoxins of pathogenic microorganisms to combat infectious diseases of various aetiologies, the staff of the SSI State Centre of Innovative Biotechnologies developed the preparation "Antracol", the basis of which is the exotoxin of *Bacillus anthracis*. The "Antracol" vaccine has demonstrated its advantages in the formation of immunity in experimental animals (guinea pigs). The "Antracol" vaccine included the life products of the virulent strain *E. coli* IVM-1, deposited in the State Scientific Control Institute of Biotechnology and Strains of Microorganisms, and the vaccine strain *B. anthracis* K-79 Z. The use of the "Antracol" vaccine is accompanied by the creation of antitoxic immunity against the causative agent of anthrax. The formation of immunity begins 3-4 hours after vaccination, due to the presence of a specific antigen (exotoxin) in the vaccine. The use of the "Antracol" vaccine to fight anthrax neutralizes the adverse impact of the disease on animal health and reduces economic losses. During an outbreak of anthrax in farms where the "Antracol" vaccine is used, the epizootic situation normalizes, and losses from the disease decrease. "Antracol" vaccine is an abacillary preparation for the rapid creation of anti-anthrax immunity in the foci of an anthrax outbreak, in field conditions when there is a threat of mass infection of animals, and for the immunization of animals for which the use of live spore vaccine is prohibited – exhausted, deep-bodied, in difficult working conditions, young animals up to 8 months old, etc. Vaccines against anthrax used in farms of Ukraine are live spore vaccines (Vaccine against animal anthrax from the strain "Sterne $34F_2$", Live spore vaccine against animal anthrax from the strain "SB", Vaccine against animal anthrax from the strain *Bacillus anthracis* UA-07 "Antravak", Vaccine against animal anthrax from strain K-79Z), is used to create active immunity against anthrax in farm animals. Immunity in animals occurs 10 days after vaccination, they are not used as emergency preparations.

Analysing world literary sources [42; 43], regarding the construction of an abacillary vaccine, it was concluded that prophylactic preparations based on *Bacillus anthracis* metabolite products are not manufactured in Ukraine.

According to the authors [43], *B. anthracis* "Sterne $34F_2$" is a strain traditionally used to produce vaccines. The authors of the "AVA" vaccine registered allergic reactions in volunteers after its use. Scientists at the SSI State Centre of Innovative Biotechnologies have proven that it has a high concentration of total protein and does not produce exotoxin in high titres, unlike *B. anthracis* K-79 Z.

As a result of the conducted studies, it was established that the *B. anthracis* K-79 Z strain does not cause an allergic reaction in laboratory animals and provides their protection at a high titre (60%).

The main factor in the reactogenicity of modern anti-anthrax vaccines is the features of the vaccine strains used in the spore suspension with the addition of preservatives. Therefore, the search for both exotoxin producers and compositions of exotoxin compounds continues [37-39], both according to indicators of an allergic reaction and reduction of post-vaccination consequences [34-36], also considering the duration of the protective effect and improving the compositions [27; 28]. Immunization of animals [29-31] shows a more efficacious influence of exotoxin as the main component of the preparation or the main ingredient of the composition [32; 33].

The "Antracol" vaccine does not contain a live spore culture of the anthrax vaccine strain and is composed of exotoxins without chemical preservatives that can cause post-vaccination complications. The study of exotoxins of vaccine strains and exotoxin as a component of the preparation is relevant and synergizes with the research of foreign scientists (USA, Great Britain, Belgium) [32; 33]. The search for the safest and most universal preparation against anthrax continues today around the world.

Conclusions

The protective function of the vaccine preparation against anthrax depends on the protective features of the vaccine strain included in its composition. The "Antracol" vaccine, which includes the extracellular toxin of *B. anthracis* K-79 Z in a titre 1 : 64 – 1 : 128, has increased protection against the pathogenic strain of *B. anthracis* 92 Z, which forms a capsule.

The lowest level of total protein was found in the exotoxin of the *B. anthracis* K-79 Z strain with a titre 1 : 128 – 0.19 mg/ml. While during the study of the exotoxin from *B. anthracis* SB, a lower concentration of total protein was noted at a titre 1 : 32 – 0.4 mg/ml, and *B. anthracis* 55 – 1 : 16 has a high concentration of total protein – 0.5 mg/ml. This characteristic of *B. anthracis* K-79 Z provides an advantage for the use of its metabolic products during the production of prophylactic preparations.

Vaccine strains *B. anthracis* $34F_2$ Sterne and *B. anthracis* 55 do not produce exotoxins in high titres according to the Ascoli reaction and have an average level of immunogenic activity.

A comparison of vaccines, spore (traditional) with the abacillary vaccine "Antracol" showed that during infection of *B. anthracis* 92 Z animals that were vaccinated, 60% of protection was registered when using prophylactic preparations from strains of *B. anthracis* SB and *B. anthracis* K-79 Z, as well as "Antracol".

Based on the study results, scientists at SSI "State Centre for Innovative Biotechnologies" can convincingly claim that the absence of spores in vaccines based on exotoxins allows solving the problems of vaccinating all types of mammals, including animals of different age groups and regardless of immune status. In the future, research on exotoxins of *B. anthracis* will be continued with the purpose of creating new anti-anthrax preparations. Continuation of the study of preparations based on exotoxins, with the purpose of improvement and implementation in the practice of veterinary medicine.

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Перспектива використання екзотоксину *B. anthracis* у конструюванні протисибіркових препаратів екстреної дії

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Анотація. Актуальність дослідження зумовлена тим, що на території України періодично реєструються спалахи сибірки, не тільки в жуйних тварин, а й в свиней, хутрових звірів, собак і людей. Мета роботи – провести дослідження захисних властивостей дослідних вакцин і абацилярної вакцини «Антракол» і довести імуногенну дію екстрацелюлярного токсину зі штаму *B. anthracis* K-79 Z. Для дослідів використовували культури вакцинних штамів антракса: *B. anthracis* 55, *B. anthracis* СБ, *B. anthracis* K-79 Z і вакцину «Антракол» (експериментальна розробка). В роботі використовували мікробіологічні, клініко-біологічні та біотехнологічні методи досліджень. Захисну дію вивчали на мурчаках (*Cavia porcellus*). Проведено гострий дослід із вірулентним штамом *B. anthracis* 92 Z. Від зазначених культур отримали екзотоксин. Його титр визначали в реакції диск-преципітації. Найвищий результат щодо продукції екзотоксину зафіксовано у *B. anthracis* K-79 Z 1 : 128 за концентрації сумарного білка – 0,19 мг/мл, тоді як екзотоксин штаму *B. anthracis* 55 з титром 1 : 32 показав високу концентрацію сумарного білка – 0,4 мг/мл. Вплив екзотоксинів *B. anthracis* на організм вивчали шляхом їх введення лабораторним тваринам в різних титрах екзотоксинів із наступним зараженням патогенним штамом *B. anthracis* 92 Z. Екзотоксин вакцинного штаму *B. anthracis* K-79 Z в титрі 1 : 64 – 1 : 128 виявляє найкращі захисні властивості проти патогенного штаму. Встановлено, що вакцинні штами *B. anthracis* СБ і B. anthracis K-79 Z володіють однаковим рівнем захисту лабораторних тварин за експериментального зараження, що становить 60 %, тоді як вакцина зі штаму *B. anthracis* 34F2 показала рівень захисту 20 %. За результатами проведених досліджень виявилося доцільним використовувати екзотоксин *B. anthracis* у розробці профілактичних препаратах щодо сибірки. Результати досліджень можуть бути використані науковцями і спеціалістами в галузі ветеринарної медицини у розробці нових та вдосконаленні наявних вакцин для ефективної профілактики сибірки *B. anthracis*

Ключові слова: сибірка, метаболіти, превентивна дія, абацилярна вакцина «Антракол»

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