Dear colleagues!

The modern trends of biological threats growing, emergent diseases (Lumpy skin disease, Foot-and-mouth disease, African swine fever, Avian influenza and other in Europe and Asia) determine the necessarily to pay the extremely high attention to the biosafety issues and biological hazards control.

The National Scientific Center 'Institute of the Experimental and Clinical Veterinary Medicine' is the leading specialized research institution in Ukraine created for solving scientific and practical tasks of veterinary animal. NSC IECVM's basic research are focused on: immunogenesis and disease pathogenesis, indications, authentications, isolations and studies of biological features of their causative agents, developments of facilities and systems of monitoring, diagnostics, prophylaxis and prognostication of infectious diseases of animals, monitoring of quality and unconcern of agricultural produce and development of the normative basis for animal diseases control and biosafety. NSC IECVM coordinates implementation of scientific researches on questions veterinary medicine, that conduct scientific establishments of NAAS, State Service of Ukraine for Food Safety and Consumer Protection, and Higher educational establishments of Ukraine of agrarian profile.

New journal 'Journal for Veterinary Medicine, Biotechnology and Biosafety", discovered in 2015, aimed to consolidate and share the new developments and achievements in the area of biological science. This was recognized as the profile edition for veterinary medicine doctors and biologists in Ukraine. Our journal promotes the research of Ukrainian institutions, publishing their achievements in English, and sharing it among the scientific community. It includes cooperative veterinary and medical aspects, fitting to One Health Approach declared by WHO, OIE, and FAO. It was included in Index Copernicus and eLibrary scientific databases.

The Editorial board hopes, that our issue will be interesting for wide auditorium of scientists and practical specialists in veterinary medicine, biology, biotechnology and biosafety. We invite new authors for fruitful collaboration and joint development.

Sincerely yours,
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GUIDELINES FOR THE PREPARATION OF THE PAPERS SUBMITTED FOR PUBLICATION AT THE 'JOURNAL FOR VETERINARY MEDICINE, BIOTECHNOLOGY AND BIOSAFETY'

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2. Papers must be written in English

3. Authors make sure there are no typographical errors in the manuscript

4. Papers must be presented in Word format, in an A4 layout, using Times New Roman 14 point font, which should be single-spaced with 25 mm margins

5. Tables and illustrations must be must be submitted as separate files and inserted in the text

6. Papers must be assembled in the following order:

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INFLUENCE OF PROBIOTICS ON HISTOSTRUCTURE OF THE BURSA OF FABRICIUS IN BROILER CHICKENS

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Summary. The article deals with the data on the influence of probiotic preparation BPS-44 and 2% yeast *Saccharomyces cerevisiae* on the histostructure of tissues of the cloacal sac (bursa of Fabricius) of broiler chickens. The conducted histological researches have shown that in chickens of the control group at the end of the experiment in the cloacal sac were revealed intraepithelial microcystic cavities, but in the medullary substance of the lymph nodes, necrotic changes are recorded and glandular structures are formed, indicating an insufficient level of lymphopoiesis.

The use of these probiotic preparations to broilers from the experimental groups during the period of their growth in the ration caused a normalizing effect on the morphostructure of the cloacal sac and, in particular, on the action of 2% yeast *Saccharomyces cerevisiae*. It is evidenced by the absence of the formation of microscystic cavities in the epithelial layer of the cloacal sac (signs of slowing down the processes of age involution). In this case, the lymph nodes are numerical, the division into cortical and medullar substance is clear, they were densely populated with lymphoid elements, indicating the possibility of forming an adequate immune response in the poultry of this group.

Keywords: probiotics, broilers, histology, cloacal sac

Introduction. At the present stage of the development of poultry farming in Ukraine it is important to ensure high profitability of production. Simultaneously, the intensive use of poultry in conditions of high density and significant influence of anthropogenic and natural factors leads to a decrease in the organism's resistance, an increase in morbidity and mortality (Lysko, 2008; Belova Gabzalilova and Topuria, 2009; Bessarabov et al., 2006; Temiraev et al., 2007). Therefore, the problem of increasing the viability of poultry is highly relevant and would allow maintaining high productivity and profitability of poultry farming.

Due to this, search for new pharmacological agents that increase the resistance and productivity of poultry has a huge economic significance. In this regard, the use of preparations containing the natural intestinal microflora — probiotics — is worthy of attention. The most important lever that regulates these processes is the specific products of the physiological microflora of the gastrointestinal tract (Subbotin, 1999; Subbotin, 1994; Subbotin and Sidorov, 1991, 1998; Nozdrin, 2003; Shevchenko, 2002; Ivanova, 2002). The researchers proved the possibility of replacing antibiotics with probiotics capable of influencing the body at the systemic level and participating in the work of regulatory systems, increasing the nonspecific resistance and resistance of young animals to diseases (Ivanova, 2007).

Along with this, it should be noted that the immunobiological reactivity of the organism of young poultry depends on a large extent on the functioning of the immune system. It is known that the feature of the immune system of chicken- broilers in the condition of intensive fattening is that they are in the stage of formation and initial development, which determines the originality of the response to antigenic stimulation and stress factors of the external and internal environment of the macroorganism.

In the poultry, there are central and peripheral lymphoid organs of the immune system. The central lymphoid organs include: the thymus, the bursa of Fabricius and the bone marrow (Bajdevljatov and Baydevlyatova, 2016; Khenenou et al., 2012). The bursa of Fabricius belongs to the lympho-epithelial organs, whose involution develops according to some data after 35 days (Krasnikov et al., 2006; Ribatti, Crivellato and Vacca, 2006), or even at a later date — in 160 and even 220 days according to others (Zharova, 2008; Mazurkevich, 2000). Probably such a rush in terms of involution may be related, in particular, to active immunization of the poultry, because this organ is responsible for the formation of humoral immunity, all processes occurring in it, are directly related to immunomorphology.

In the scientific literature, the investigation of the influence of probiotic preparations on the morphological

structure of immunocompetent organs, and in particular the cloacal sac are fragmentary and require detailed study.

Due to this, the purpose of the presented work was to elucidate the influence of the preparation BPS-44, made on the basis of the production strain of *Bacillus subtilis* bacteria and 2.0% yeast *Saccharomyces cerevisiae* on the histostructure of tissue of broiler chickens cloacal sac.

Materials and methods. The research was carried out on broiler chickens of the ROSS-308 cross, grown in one of the farms of the Lviv Region. The housing conditions of the chickens was standard, with free access to feed and water. Technological parameters of broiler rearing (temperature and light regime) were in accordance with the norms. Experiments were conducted in three groups of broiler chickens of 100 heads in each as shown in Table 1.

Table 1 — Scheme of experiment

Groups	Name of the preparation	Scheme of preparation use	Age of the poultry, days
Control	No preparations were prescribed	—	4–43
Experimental 1	BPS-44	Three courses for 7 days in a row with 7 daily breaks	5–11 21–27 36–42
Experimental 2	*Saccharomyces cerevisiae* 2%	Constantly	4–43

The control group of broilers was fed the standard feed (SC) in accordance with the existing norms recommended for the ROSS-308 cross; the 1st experimental group in addition to the standard feed received a probiotic BPS-44 (registration certificate No. 2154-04-0254-06 dated 24 November 2006), made on the basis of the production strain of bacteria *Bacillus subtilis* ssp. *subtilis* 44-p, dose 0.21 g/kg, the 2nd experimental group — 2.0% of yeast *Saccharomyces cerevisiae.*

Vaccination of broilers was carried out in accordance with the scheme shown in Table 2.

Table 2 — Vaccination scheme

Age, days	Vaccines	Method of input
11	BRONHIKAL® I SPF (Croatia)	watering
13	BIO-VAC La-Sota (Italy)	
15	GUMBOKAL IM FORTE SPF (Croatia)	

At the end of the experiment, which corresponded to the completion of the period of poultry breeding, was carried out the autopsy using the Shore method.

During the autopsy, pieces of the immune system organs, and in particular tissues of the cloacal sac for histological searches, were taken.

The material was fixed in 10.0% of neutral formalin solution and Buena liquid. The histological sections were made with the help of a microtome, were colored with hematoxylin-eosin, methyl-green and pyronin G according to Brach (Merkulov, 1969; Roskin and Levinson, 1957).

Results. Objective and correct method of morpho-physiological changes indications, occurring in the body under the actions of various environmental factors at the cellular-tissue level is the histological searches of the lymphoid tissue of the immune system.

For histological searches of chickens cloacal sac in the control group on many areas, the epithelial layer was infiltrated with lymphocytes, necrotic changes of epithelial cells were recorded, as a result of which, separate epithelial cells were desquamated into the lumen of the cloacal sac. Occasionally moderate proliferation of the epithelium was observed (Fig. 1).

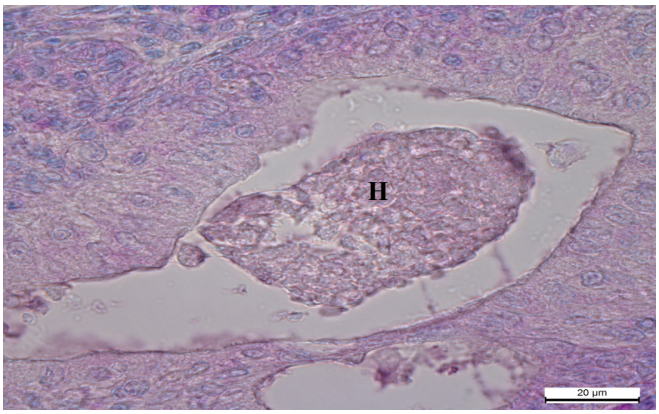

Figure 1. Cloacal sac. Necrotic changes in the structural elements of the medullary substance of the lymph nodes. H — structural elements of the medullary substance of the lymph nodes. Methylene-green and pyronin for Brashe ×1000. Control group.

Microscopic cavities of various sizes were formed on many parts of the epithelial layer. The individual vessels of the mucous membrane itself were enlarged, filled with red blood cells and lymphocytes. Moderate infiltration of the loose connective tissue of the mucous membrane with plasma cells, lymphocytes, granulocytes, tissue basophils were recorded.

Cellular composition of lymphoid nodes was represented mainly by B-lymphocytes, as well as T-lymphocytes, lymphoblasts, prolifocytes, plasmacytes, macrophages and granulocytes. Among lymphocytes there were small, medium and large.

The medullary substance of most lymphoid nodules in the control poultry group contained a small amount of cellular elements (Fig. 2).

In certain lymphoid nodules, marked necrotic changes were developing, especially in the medullary substance. As a result, the central part of some lymphoid nodes were

filled with necrotic detritus. In the center of individual lymphoid nodes glandular structures were formed, which is a sign of inhibition of lymphopoiesis. The proliferation of lymphoblasts in lymphoid nodes was not significant. Lymphatic vessels of the stroma are expanded, lymphatic vessels were full. There was also an enlargement of the blood vessels that were full of red blood cells and single granulocytes. Occasionally there were perivascular edemas.

In some capillaries, red blood cells were in several rows, it is noted that they were glued, indicating the development of a stasis. Necrotic changes in individual endothelial cells were observed. In some vessels, slightly expressed proliferative processes of the endothelium were recorded. In adventitia, there is proliferation of pericytes. As well, there were perivascular infiltrations from plasma cells and lymphocytes.

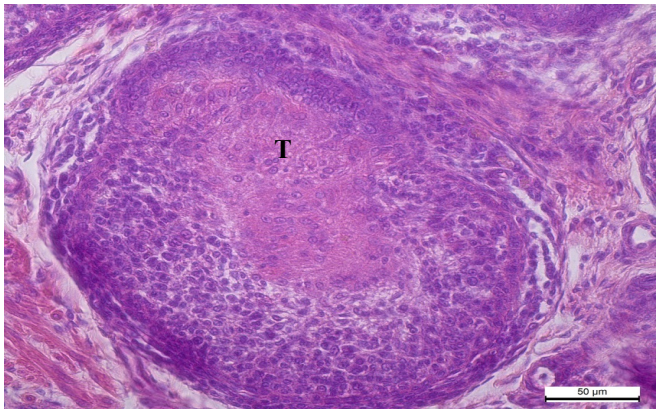

Figure 2. Cloacal sac. T —necrotic detritus in the central part of the lymphoid nodule ×400. Control group.

Separate vessels of the muscle membrane were enlarged, filled with red blood cells and isolated granulocytes. Intramuscular connective tissue was somewhat swollen. The serous membrane was formed by a basic plate containing a slightly swollen loose connective tissue covered with a mesothelium.

The mucous membrane of the cloacal sac was lined with a simple columnar epithelium, which in some areas went into a simple multi-row, contained a smooth groove (Fig. 3).

Somewhere epithelial proliferation was observed. Necrotic changes of epithelial cells were also registered, as a result of which epithelial cells are desquamated into the lumen of the cloacal sac. Microcystic cavities were formed on many parts of the epithelial layer, some of which contained a moderate amount of weakly basophilic content. Infiltration of the epithelial layer by lymphocytes was noted. Moderate infiltration of the mucous membrane by the cells of the lymphoid series, including plasmocytes, was observed. In the connective tissue of the mucous membrane and submucosal membrane, tissue basophils, as well as plasmocytes, single lymphocytes and granulocytes were visualized.

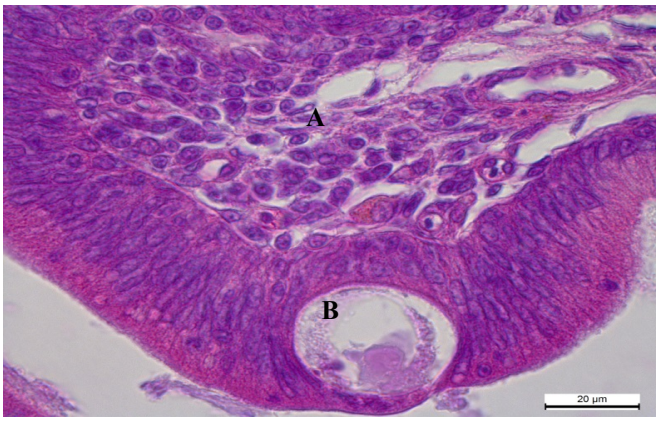

Figure 3. Cloacal sac. Intraepithelial cyst. Infiltration of the submucosal lymphocytes. A — infiltration of the submucosal membrane lymphocytes. B — intraepithelial cyst. Hematoxylin-eosin ×1000. Control group.

Lymphoid nodules were found in the organism of the broilers of the group fed probiotic preparation based on *Bacillus subtilis* ssp *subtilis* in the folds of the cloacal mucous membrane (Fig. 4). The division of most lymphoid nodes into cortical and medullary substances was preserved. There was a depletion of the medullary substance of most lymphoid nodes by cellular elements. In addition, in some lymphoid nodes, necrotic changes in the structural elements of the medullary substance were developed. As a result, the central part of some lymph nodes was filled with necrotic masses. Sometimes glandular structures were formed in the medullary substance, indicating the inhibition of lymphopoiesis. The proliferation of lymphoblasts in lymphoid nodes was expressed slightly. The number of cellular elements in the cortical substance was moderate. Occasionally there were necrotized lymphocytes. The number of macrophages that phagocyte the remains of necrotic cells increased.

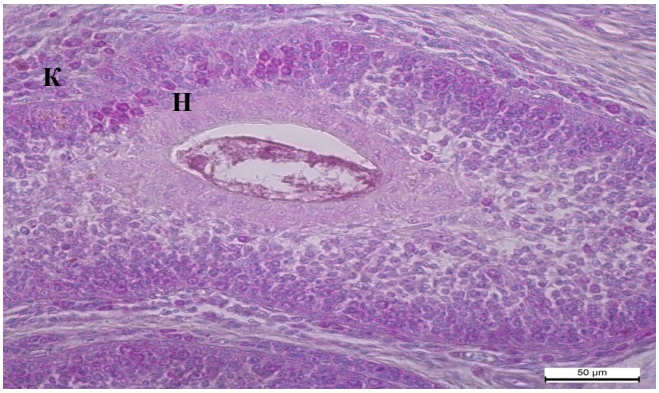

Figure 4. Necrotic changes in the structural elements of the medullary substance of the lymph nodes. Moderate amount of B-lymphocytes in the cortical substance of the lymph nodes. H — necrotic changes of structural elements of the medullary substance, K — B-lymphocytes in the cortical substance of the lymph nodes. Methylene-green and pyronin for Brashe ×400. Experimental group 1.

There was perivascular edema. In adventitia, there was proliferation of pericytes. which indicates the course of processes of physiological regeneration in tissues and the formation of new capillaries. There were available perivascular infiltrations from plasma cells, lymphocytes and single tissue basophils. Connective tissue around the hyperemic vessels were slightly swollen.

When carrying out the histological characteristics of the cloacal sac of chickens- broiler under the action of 2.0% yeast *Saccharomyces cerevisiae* it was found that the mucous membrane of the cloacal sac was uneven, within one fold the inequalities of the epithelial layer were intensified (Fig. 5). There was a proliferation of the epithelium, and in some areas moderate hypersecretion of mucus. Necrotic changes in epithelial cells were rare. Microscopic cavities in the epithelial layer did not occur. Moderate infiltration of the mucous membrane by the cells of the lymphoid series was observed. Plasma cells predominated in the specified cell infiltrates.

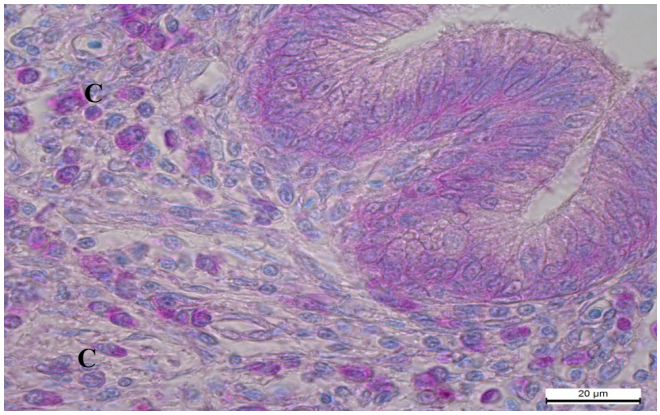

Figure 5. Cloacal sac. Significant amount of plasma cells in the connective tissue of the mucous membrane. C — plasma cells in the connective tissue of the mucous membrane. Methylene-green and pyronin for Brashe ×1000. Experimental group 1.

Lymphoid nodes were located in the folds of the cloacal mucous membrane, and in most lymphoid nodes the division into medullary and cortical substance was distinct (Fig. 6).

The cellular composition of the lymphoid nodules remained unchanged for the introduction of 2.0% yeast *Saccharomyces cerevisiae* with the predominance of B-lymphocytes in the structure of the tissue. The proliferation of lymphoblasts in lymphoid nodes was well expressed. The number of cellular elements in the cortical substance was significant. Only in certain lymphoid nodes in the medullary substance, and to a lesser extent, in the cortical substance, there were single necrotized lymphocytes.

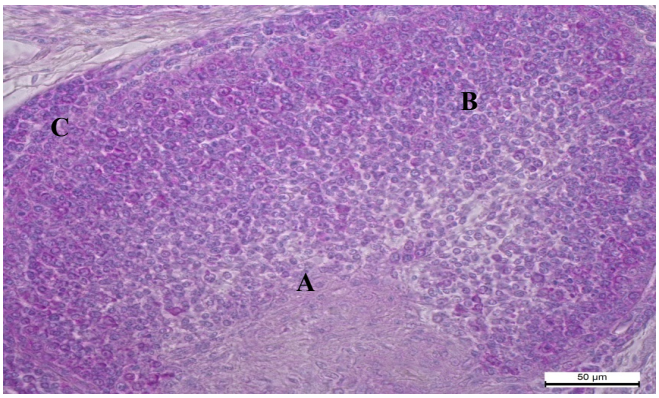

Figure 6. Cloacal sac. Clear division of lymph nodes into cortical and medullary substances. Moderate amount of cellular elements in the cortical substance of the lymph nodes. A — medullary substances, B — B-lymphocytes, C — cortical substance. Hematoxylin-eosin ×400. Experimental group 2.

There was an enlargement of the blood vessels that were full of red blood cells and single granulocytes. There were also perivascular infiltrates that consisted mainly of plasma cells and lymphocytes. The individual vessels of the muscle were slightly enlarged, containing red blood cells and single granulocytes. No pronounced perivascular edema was detected. The subserous basis, which is also formed by a loose connective tissue was poorly expressed.

Conclusions. As a result of the histological search of the central organ of the immune system of broiler chickens it was found that in the poultry of the control group, which were fed with standard combined feed in the cloacal sac intraepithelial microvascular cavities were developed, and in the medullary substance of the lymph nodes necrotic changes were recorded and glandular structures were formed, indicating an insufficient level of lymphopoiesis.

In the poultry of the experimental group 1, which were fed with the probiotic BPS-44 in the epithelial layer of the cloacal sac, isolated single microcystic cavities were detected, and necrotic changes in the medullary substance of the lymph nodes were recorded.

The most optimal morphological state of the cloacal sac was found in the chickenss of the second experimental group, which were fed with 2% yeast *Saccharomyces cerevisiae* in the composition of the mixed fodder. In particular, we did not register the formation of microscopic cavities in the epithelial layer of the cloacal sac, lymph nodes were numerical, the division into cortical and medullary substance was clear, they were densely populated with lymphoid elements, which indicate the possibility of forming of complete immune response in the poultry of this group.

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DETERMINATION OF THE VITAMIN-MINERAL PREPARATION 'ENERGOLIT' STABILITY FOR THE TREATMENT OF METABOLIC DISORDERS IN ANIMALS

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Summary. The aim of experiments was the study the shelf life of the injectable preparation 'Energolit' when stored in vials of light glass. Studies were carried out at a temperature within from +10 to +25 °C and relative humidity (60 ± 5%). Experiments were carried out in 3, 6, 9, 12, 18, 24 months, and one day after the expiration date after the puncture of the stopper with a sterile injection needle.

The control was the newly prepared preparation. We checked organoleptic parameters, pH, microbial contamination to assess the quality of the 'Energolit'. In addition, we determined the quantitative content of vitamins B_1, B_2, B_3, B_5, B_6, and B_{12} by methods described in the 'State Pharmacopoeia of Ukraine'.

Three tested batches of the preparation 'Energolit' showed constant composition throughout all period of study during two years of storage, as well as one day after the puncture of the stopper with a sterile injection needle in the primary packaging. Experimental samples of the preparation were within possible variation during the tests on changes of pH, identity and content of the vitamins B_1, B_2, B_3, B_5, B_6, and B_{12}. Microbial contamination (*Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa*) was not detected. Fungi contamination were not detected during the experiment. Fungi contamination was less than 10 CFU/ml one day after the puncture.

The optimal storage terms of the preparation has been determined. The findings indicated, that the shelf life of the preparation 'Energolit' is 2 years. The pH and mass fraction of the vitamins B group were unchanged or were within the limits of possible variation in the tested samples during the experiment. Microbial contamination was not detected.

Keywords: B vitamins, stability, shelf life, 'Energolit'

Introduction. Despite the significant amount of therapeutic agents for the treatment of metabolic disorders in the animal, the pharmaceutical market in Ukraine feels the need for modern, complex and affordable preparations. The present realities led to the lack of planning and systematic veterinary and zootechnical measures in the private sector. Farmers expects at veterinarian to solve all problems with one injection. Therefore, the promising direction of veterinary pharmacology is the combination of multicomponent preparations. They have a wider range of applications due to the synergy and compatibility of the combined components.

Therefore, at the request of the veterinary preparations market and with the purpose of import substitution, Private Enterprise 'Biopharm', together with the Rivne Research Station of Epizootology of the Institute of Veterinary Medicine of the NAAS, developed a new vitamin and mineral preparation 'Energolit'. The application of that preparation optimizes the cost, reduce the timing of medical procedures and simplify therapeutic manipulations.

The composition of the product includes vitamin B complex, glucose, sorbitol, sodium salts, potassium, calcium, magnesium and amino acids (arginine, glutamic acid, lysine, and methionine). 'Energolit' is recommended for the treatment of metabolic disorders of various etiologies, including ketosis and acidosis; hepatitis and toxicosis, in the period of recovery after prior diseases; for dehydration of the body from diarrhea, vomiting and blood loss.

The preparation is used for weakened and depleted animals to normalize metabolism during pregnancy and lactation, for increase resistance to physical activity and stress, and also to horses and dogs — before intensive work, competitions and long-term transportation (Sachuk, 2018).

A prerequisite for the registration of new preparations is the study of their stability in storage. The study of the expiration date of new preparations in order to obtain information on changes in their quality over time under the influence of environmental factors (temperature, humidity, illumination, etc.) is an obligatory type of scientific research (Kotsiumbas et al., 2006).

The aim of the work was to study the shelf life of the injectable preparation 'Energolit' when stored in vials of light glass.

Materials and methods. Investigation of the injection preparation 'Energolit' was conducted on three pilot-industrial series (010216, 010316, 010416) made on LLC 'DEVIE' (Ukraine). The tests were carried out on as packaged preparation (a vial of light glass II-250-B-2 according TUU (Technical Specifications of Ukraine) 26.1-04763746-025:2008; a cap of aluminum with perforation type K-3-28; rubber cork for block, type 28-B3). To determine the shelf life of the preparation stability, studies were conducted at recommended storage conditions: at a temperature from +10 to +25 °C and

relative humidity (60 ± 5%), in periods: 3, 6, 9, 12, 18, and 24 months. And one day after the end after the piercing of the stopper with a sterile injection needle. The test sample was a newly prepared preparation.

According to normative requirements and literary sources, during the test, the fundamental properties of the preparation were determined: identification of active substances, control of the basic physical indicators and the most accurate analytical techniques for their determination were selected. In the case of 'Energolit', the organoleptic parameters (appearance, color, precipitate, etc.), pH, presence of microbial contamination were determined, and the quantitative content of vitamins B_1, B_2, B_3, B_5, B_6, and B_{12} was identified and determined (Kotsiumbas et al., 2006; USPCQM, 2014–2018; Tykhonov and Yarnykh, 2016).

The study of microbiological purity was carried out by direct inoculation method according to the 'State Pharmacopoeia of Ukraine' (SPU) procedure (USPCQM, 2014–2018).

Microbiological limit tests of the injection solution included the quantification of viable bacteria and fungi, as well as the identification of certain types of microorganisms not available in sterile medicinal products. Inoculation of medium for the next dilution of the preparation: medium No. 1 — 1:10, medium No. 2 — 1:10, medium No. 3 — 1:10, medium No. 4 — 1:20. For this, 1.0 ml of the preparation was shaken with 10 ml of phosphate buffer (pH 7.0). 1 ml of the resulting solution of the preparation was inoculated on a liquid and solid Sabouraud's medium, meat infusion broth containing 2% glucose, a thioglycollate medium and a blood agar. All inoculated medium was incubated at a temperature of 30–35 °C. Daily, as well as after the end of the incubation period, inoculations were examined in diffused light. As a control, nutrient media were used without adding the preparation. In addition, studies have been conducted on the presence of bacteria *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa* (USPCQM, 2014–2018).

The determination of the identity of vitamins B_1, B_2, B_3, B_5, B_6, and B_{12} was carried out by liquid chromatography method, comparing the peak time of B vitamins in chromatograms of Work Solution Reference Standards (WSRS) and peaks corresponding to vitamins B_1, B_2, B_3, B_5, B_6, and B_{12} on the chromatogram of the work solution of the test sample (WSTS). The difference in peak time did not exceed the relative standard deviation calculated for WSRS from five consecutive chromatograms, but not more than recommended by the SPU (USPCQM, 2014–2018).

Determination of the quantitative content of vitamins B_1, B_2, B_3, B_5, B_6, and B_{12} was carried out by high performance liquid chromatography on a liquid chromatograph LPP-205 (Czech Republic) according to the following parameters:

— chromatographic tube 250×4.6 mm;

— Luna® Omega 5 μm sorbent Polar C18 100 Å;

— mobile phase — a mixture of acetonitrile with a buffer solution;

— flow rate of the mobile phase — 1.0 ml/min;

— detection at wavelengths of 195/265 nm (vitamins B_3, B_5, and B_{12} — 195 nm; B_1, B_2, B_6 — 265 nm);

— tube temperature +25 °C;

— registration scale — 0.5 absorbance units;

— signal integration — computer integration;

— volume of injection — 0.010 ml.

The content of each of the vitamins in the preparation 'Energolit' is calculated by the ratio of the areas of the corresponding peaks on the chromatograms of the test sample and standard samples.

For the investigated samples quality indicators had to meet specifications and standards specified in Table 1.

Table 1 — Quality control indicators

Name of the indicator	Specifications and standards
Description	Orange solution
Packing volume	250 ml ± 10.0%
pH	5.0–7.0
Identity of vitamins B_1, B_2, B_3, B_5, B_6, B_{12}	Positive
Quantity of vitamin B_1	0.1 mg/ml ± 10.0%
Quantity of vitamin B_2	0.02 mg/ml ± 10.0%
Quantity of vitamin B_3	1.5 mg/ml ± 10.0%
Quantity of vitamin B_5	0.05 mg/ml ± 10.0%
Quantity of vitamin B_6	0.15 mg/ml ± 10.0%
Quantity of vitamin B_{12}	0.03 mg/ml ± 10.0%
Sterility	Must be sterile

Statistical processing of the obtained results was carried out in accordance with the SPU (n = 3) (USPCQM, 2014–2018; Belikov, 1985; Rokitskiy, 1973).

Results and discussion. Table 2 presents the results of experimental studies of stability, covering the physicochemical and pharmacological performance of three experimental and industrial series of vitamin and mineral preparation 'Energolit'. It was confirmed that during the two years of storage, as well as one day after the piercing of the stopper with a sterile injection needle, in the primary packaging at a temperature of from +5 to +25 °C and relative humidity (60 ± 5%), the three studied series of the preparation 'Energolit' showed stability of composition.

During the tests on changes of pH, identity and content of the vitamins B_1, B_2, B_3, B_5, B_6, and B_{12} of the experimental samples of the preparation were within possible variation. Microbial contamination (*Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa*) was not detected. Fungi contamination was not detected during the study as well. In one day after the puncture contamination was less than 10 CFU/ml.

Table 2 — Experimental results of the preparation 'Energolit' stability, when stored in glass vials (M ± m, n = 3)

Control period, months	Drug quality indicators															
				Vitamin B_1		Vitamin B_2		Vitamin B_3		Vitamin B_5		Vitamin B_6		Vitamin B_{12}	
	Description	pH	Microbial contamination, CFU/ml	Identity	Quantitative determination, mg/ml	Identity	Quantitative determination, mg/ml	Identity	Quantitative determination, mg/ml	Identity	Quantitative determination, mg/ml	Identity	Quantitative determination, mg/ml	Identity	Quantitative determination, mg/ml
0	C	5.5± 0.04	C	P	0.103± 0.001	P	0.023± 0.0004	P	1.53± 0.004	P	0.05± 0.007	P	0.15± 0.0004	P	0.03± 0.0004
3	C	5.5± 0.04	C	P	0.102± 0.0008	P	0.022± 0.0004	P	1.52± 0.004	P	0.05± 0.0004	P	0.15± 0.0004	P	0.03± 0.0004
6	C	5.6± 0.04	C	P	0.102± 0.0007	P	0.022± 0.0004*	P	1.51± 0.007	P	0.05± 0.0004	P	0.15± 0.0004**	P	0.03± 0.0004
9	C	5.6± 0.04	C	P	0.101± 0.0004	P	0.021± 0.0004*	P	1.50± 0.004*	P	0.05± 0.0007*	P	0.15± 0.0004**	P	0.03± 0.0008
12	C	5.6± 0.04	C	P	0.101± 0.0004	P	0.021± 0.0004**	P	1.49± 0.004**	P	0.05± 0.0007*	P	0.15± 0.0012*	P	0.03± 0.0008
18	C	5.5± 0.04	C	P	0.100± 0.0004	P	0.019± 0.0004**	P	1.48± 0.007**	P	0.05± 0.0004**	P	0.15± 0.0012	P	0.03± 0.0004*
24	C	5.6± 0.04	C	P	0.099± 0.0007*	P	0.019± 0.0004**	P	1.48± 0.011*	P	0.05± 0.0008*	P	0.15± 0.0015	P	0.03± 0.0007*
1 day after piercing	C	5.7± 0.04*	C	P	0.099± 0.0011	P	0.018± 0.0008**	P	1.48± 0.015*	P	0.05± 0.0011**	P	0.15± 0.0025	P	0.03± 0.0011*

Notes: * — $p < 0.05$; ** — $p < 0.01$ in comparison before control; C — corresponds; P — positive.

It should be noted that the preparation meets SPU indicator for microbiological purity. The spectrophotometric studies of the samples compared with the initial data confirm the results that the spectra of the studied samples are not significantly different.

Thus, the results of the results of stability studies indicate the rationality of the composition and manufacturing technology of the preparation. The components are selected according to the technological purpose, physical, chemical and functional characteristics.

Conclusions and prospects for further research. The optimal terms of storage of the preparation when stored in vials of light glass in consumer packaging — at a temperature of up to 25 °C and a relative humidity of 60 ± 5% that meets our climatic conditions has been determined. The obtained experimental data indicated that the shelf life of the preparation 'Energolit' is 2 years. The pH and mass fraction of the vitamins B group were unchanged or within the possible variation in the tested samples during the study, and microbial contamination was not detected. The research results are included in the new preparation registration dossier.

Further research will be focused on determination the experimental samples of the injectable preparation 'Energolit' specific activity in the conditions of an extended shelf-life.

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STUDY OF RAPPORT OF THE MILK POLLUTION BY MULTIDRUG-RESISTANT BACTERIA ('SUPERBUGS'), COWS MORBIDITY AND CALVES LETHALITY IN HOLDINGS OF EASTERN UKRAINE

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Summary. *Pasteurella multocida* (46.0%), *Neisseria* spp. (44.0%), *Aspergillus fumigatus* (10.0%) were isolated in stable consortia form from milk samples (n = 60) from cows in 6 herds (n = 627 heads) with clinical signs of immunodeficiency status. The bacteria of these consortia is strong resistant to lots of antibiotics from synthetic penicillin group (ampiox, amoxiclav, ampicillinum), cephalosporins (cefalexin, cefixime), and macrolides (macropene, tylosin). The etiological structure of bacterial infections in cattle and their calves (n = 57) in mentioned farm holdings of eastern Ukraine was determined (6 farms in 3 regions). In the majority of these livestock, respiratory syndrome has been recorded for a long time in 56.0% of calves from 20 days to 5 months old. The signs of diarrhea were revealed in 12.0% of lactating and dry cows of these livestock, 18.0% of these animals showed the clinical manifestation of mastitis and 9.0% of them had the diarrhea syndrome. *Pasteurella multocida* (48.0%), *Mycoplasma bovis* (32.0%), *Neisseria* spp. (12.0%), *Clostridium perfringens* (8.0%) were identified using microbiological methods from nasal and rectal swabs from calves of different age groups, from cows with clinical signs of mastitis were isolated *Mycoplasma bovis* (50.0%), *Pasteurella multocida* (34.0%), *Neisseria* spp. (16.0%). *Pasteurella multocida*, *Neisseria* spp. and *Clostridium perfringens* isolates showed strong resistance to ampiox, amoxiclav, ampicillinum, cefalexin, cefixime, macropene, and tylosin. It was concluded that mentioned multidrug-resistant animal infectious agents in human food chain could be a sources of antibiotic resistance genes for human pathogens.

Keywords: antimicrobial drugs, bacterial infections, mastitis, microbiological methods, respiratory syndrome, resistance

Introduction. Industrial technology for the production, growing and fattening of young cattle is associated with a high concentration of animals of the same age in confined areas, which contributes to the emergence of epizootic and enzootic diseases.

Bovine pneumoenteritis is the most common diseases, which is widespread among calves and causes large economic losses. Its etiological agents are viruses belonging to the families of Herpesviruses (the virus of infectious bovine rhinotracheitis), Paramyxoviruses (parainfluenza virus-3 and respiratory syncytial virus), Pestiviruses (bovine viral diarrhea-mucosal disease virus), Adenoviruses, etc. Young animals are especially seriously ill when two or more pathogens are involved in the pathological process. There are no clinical signs in animals with normal functioning immune system (Sidorenko, 1998).

In severe course of viral infection, along with the affection of sensitive cells, there is a significant inhibition of the both cellular and humoral parts of immune system, resulting in activation of conditionally pathogenic microflora (*Pasteurella*, *Mycoplasma*, *Neisseria*, *Clostridium*) and in developing of infectious process, which leads to a significant withdrawal of diseased animals and lowering their productivity (Bennett, 2008).

The bovine pneumoenteritis in calves is obligatory associated with morbidity of cows on mastitis and endometritis which are important items of the bovine pathology. The last significantly reduces the efficiency of the animals using, and it affects the economy of dairy and cattle breeding. Important role in the mastitis and endometritis pathogenesis complication plays infectious rhinotracheitis and viral diarrhea agents, when their consortia with *Pasteurella* spp., *Mycoplasma* spp., *Neisseria* spp., *Clostridium* spp., *Candida* spp. and/or *Aspergillus* spp. are formed (Demain and Sanchez, 2009).

The knowledge in etiology of the bovine pneumoenteritis and dairy cow's mastitis/endometritis and especially in antibiotic resistance of their agents is crucial for control measures development in veterinary aspects. However, it has great significance also in 'One-Health' programs aspects for control of antibiotic contamination of human food chains.

The purpose of research. To study the etiological structure of the mentioned diseases of calves and cows in farms and their relation with multidrug-resistant microflora in drinking milk in eastern Ukraine.

Materials and methods. Eighty samples of blood serum from calves, 57 samples of pathological material from dead calves (from 20 days to 6 months), 36 swabs from the rectum, 28 swabs from the nasal cavity, 50 samples of milk from cows with mastitis, and 67 vaginal swabs were used for the studies. Samples were obtained from calves with pneumoenteritis and cows with clinical signs of endometritis and mastitis in 6 farms of 3 Ukrainian regions in 2016–2017.

As it is prescribed by Standard operating procedures of National Scientific Center 'Institute of Experimental and Clinical Veterinary Medicine' (SOP NSC 'IECVM'), bacterial pathogens were isolated in form of bacterial associations/consortia as result of clinical studies (during sampling of nasopharyngeal and rectal erosion, blood or blood serum collection) and pathological investigations of dead calves were conducted (Golovko, 2007). The study of cultural and morphological properties of the separate bacteria strains were conducted using 'HiMedia' and 'Oxoid' bacteriological nutrient media listed below: meat-peptone broth with the addition of 1.0% glucose (MPB), 2.5% meat-peptone agar with 1.0% glucose (MPA), Endo agar, Olkenitsky nutrient medium, Simon's citrate agar, Muller's medium, Ploskirev's medium with synthomycinum, Bismuth-sulfite agar, PPLO selective media for *Mycoplasma* (modified Edward medium), wheat-yeast agar with the addition of 10.0% of milk serum, Hottinger broth and Hottinger nutrient agar, Sabouraud dextrose agar for fungi species isolation, Thioglycollate broth (USP Alternative) for anaerobic species isolation. The hemolytic properties of the separate bacterial isolates were studied during cultivation on MPA with the addition of 0.5% of the defibrinated sheep blood.

The pathogenicity of all isolated and identified bovine bacterial agents was evaluated by classical bioassays test on nonlinear white mice weighing 18–20 g. Antibiotic susceptibility of isolated bacterial isolates was determined by the routine disc diffusion test.

The appropriate field samples were investigated by standard virological methods as prescribed by the SOP NSC 'IECVM'.

The evaluation of the diseased calves and cows functional state was carried out by the assays of traditional clinical and biochemical parameters in their blood according to standardized methods (Samarina, 2002).

Results and discussion. Data of an epizootological survey of livestock farms and a clinical examination of cattle and progeny showed that calves pneumoenteritis is recorded in all studied Ukrainian regions.

Twin peaks of the disease incidence of calves was recorded in the winter-spring and summer periods, which is due to the massive calving of the cows (congestion), the birth of the weakened calves, with reduced resistance and bad adaptive ability. In summer, the infection was detected among 4–6 month old animals, which got sick during the winter and spring period, that is, recurrence of the chronic diseases were detected.

Associative viral-bacterial infections which manifested as pneumoenteritis in calves, dairy cow mastitis, endometritis and winter diarrhea were widespread in the structure of animal's morbidity and death on 6 studied farms (Table 1).

All studied bovine herds (n = 6) had the immunodeficiency status according to results of biochemical blood analysis.

Table 1 — The bovine morbidity and lethality structure (n = 627)

Stage	Bovine ages / group	Morbidity, %	Lethality, %
1	Calves to 20 days age, n=73	27.0–39.0	21.0–33.0
2	Calves to 2 month age, n=148	18.0–42.0	14.0–28.0
3	Calves to 3–6 month age, n=86	31.0–56.0	10.0–24.0
4	Lactating cows with clinical manifestation of mastitis, n=51	14.0–18.0	—
5	Post-partum cows with clinical manifestation of endometritis, n=22	12.0	—
6	Dry and lactating cows with clinical signs of 'winter diarrhea', n=18	8.0	1.0

The structure of etiological microflora of the bovine morbidity-mortality in 6 studied agro holdings is presented at Table 2.

Table 2 — The associative bovine bacterial infections structure

Stage	Bovine ages / group, samples	Bacterial agents proportions, %
1	Calves to 2 month age (n=34)	*Pasteurella multocida* — 45.0%; *Mycoplasma bovis* — 28.0%; *Neisseria* spp. — 17.0%; *Clostridium perfringens* — 10.0%
2	Calves to 3–6 month age (n=17)	*Pasteurella multocida* — 48.0%; *Mycoplasma bovis* — 32.0%; *Neisseria* spp. — 12.0%; *Clostridium perfringens* — 8.0%
3	Lactating cows with clinical manifestation of mastitis (n=51)	*Mycoplasma bovis* — 50.0%; *Pasteurella multocida* — 34.0%; *Neisseria* spp. — 16.0%
4	Post-partum cows with clinical manifestation of endometritis (n=27)	*Pasteurella multocida* — 46.0%; *Neisseria* spp. — 44.0%; *Aspergillus fumigatus* — 10.0%
5	Dry and lactating cows with clinical signs of 'winter diarrhea'(n=6)	Concurrent coronaviral-enterobacterial microflora

As microbiological results revealed the leading etiological role in bacterial consortia belongs to the

Pasteurella multocida (from 45.0% to 48.0%) in association with *Mycoplasma bovis* (from 28.0% to 50.0%) which has been isolated in 6 surveyed farms from all age groups of cattle. The *Neisseria* spp., *Clostridium perfringens*, *Aspergillus fumigatus* we can accept as opportunistic bacteria which support the existence of a stable association in animals. It should be noted that the proportion of *Mycoplasma bovis* in bacterial consortia reached up to 50.0% while *Pasteurella multocida* with *Neisseria* spp. were in minority in cows with mastitis. The activation of *Aspergillus fumigatus* with *Pasteurella multocida* and *Neisseria* spp. was observed in cows with endometritis. The sensitivity of the isolated cultures of microorganisms to antibiotics was determined by standard disk diffusion test (DDT) simultaneously with all the bacterial agents forming the bacterial consortia and supporting simultaneous growth on appropriate solid nutrient media.

The analysis of the microorganisms sensitivity to antibiotics determination showed that the highest activity to isolated from dead calves microorganisms (*Pasteurella multocida*, *Mycoplasma bovis*, *Neisseria* spp., *Clostridium perfringens*) was shown to tetracycline, doxycycline, marbofloxacin, ofloxacin, nitroxolin, lincomycin, spectinomycin, oxytetracycline, sulfadimezin, and trimethoprim.

The bacterial association of *Pasteurella multocida*, *Mycoplasma bovis*, *Neisseria* spp., and *Clostridium perfringens* showed resistance to antimicrobial drugs of the synthetic penicillin group (ampiox, amoxiclav, ampicillin), cephalosporins (cefalexin, cefixime), and macrolides (macropene, tylosin).

Approximately the same microflora with the same multidrug resistance was isolated from 74.0% samples of raw milk collected in 6 studied agro holdings (Table 3).

Table 3 — Bacterial profile of the raw milk samples from agro holdings in Kharkiv, Poltava, and Sumy regions

Stage	Raw milk samples		Proportion of bacteria in consortia, %				Drug-resistance of consortia by circle diameter in DDT*) to antibiotics, mm		
	Sources	Quantity	*Pasteurella multocida*	*Mycoplasma bovis*	*Neisseria* spp.	*Clostridium perfringens*	Penicillin Group	Cephalosporin Group	Macrolides
1	Holding #1, Kharkiv region	10	46	38	16	0	8–12	10–14	10–13
2	Holding #2, Kharkiv region	10	36	52	8	4	9–10	7–12	9–15
3	Holding #1, Poltava region	10	25	45	23	7	9–13	8–14	10–12
4	Holding #2, Poltava region	10	18	60	22	0	9–12	10	14–16
5	Holding #1, Sumy region	10	35	49	16	0	9–11	10–13	11–13
6	Holding #2, Sumy region	10	23	57	19	0	9–12	8–12	9–11

Notes: to 13 mm — the pathogen is not sensitive; 13–18 mm — moderately sensitive; more than 18 mm — sensitive to antibiotics; *)DDT — disk diffusion test with commercial disk kits.

Data presented in Table 3 proves that bacterial consortia structure in milk samples practically are the same as in swabs from cows and diagnostic samples from calves. The formation of multidrug-resistant microflora ('superbugs') in cattle and milk is a result of wide antibiotic spectrum application for treatment in all studied agro holdings.

Conclusions. 1. *Pasteurella multocida* (46.0%), *Neisseria* spp. (44.0%), *Aspergillus fumigatus* (10.0%) were isolated in stable consortia form in milk samples (n = 60) from cows in 6 herds (n = 627 heads) with clinical signs of immunodeficiency status. The bacteria of these consortia showed strong resistance to different antibiotics from the synthetic penicillin group (ampiox, amoxiclav, ampicillin), cephalosporins (cefalexin, cefixime) and macrolides (macropene, tylosin).

2. *Pasteurella multocida* (48.0%), *Mycoplasma bovis* (32.0%), *Neisseria* spp. (12.0%), *Clostridium perfringens* (8.0%) were identified using microbiological methods from nasal and rectal swabs from different age groups of calves as well as *Mycoplasma bovis* (50.0%), *Pasteurella multocida* (34.0%), *Neisseria* spp. (16.0%) were isolated from dairy cows with mastitis. *Pasteurella multocida*, *Neisseria* spp. and *Clostridium perfringens* isolates exhibited strong resistance to ampiox, amoxiclav, ampicillinum, cefalexin, cefixime, macropene, and tylosin.

3. Application of wide antibiotic spectrum for bovine treatment in all of studied agro holdings causes the formation of multidrug-resistant microflora ('superbugs') in animals and milk from dairy cows. Multidrug-resistant animal infectious agents in human food chain may be sources of antibiotic resistance genes for human pathogens.

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MONITORING OF DERMATOPHYTOSIS INCIDENCE IN DOMESTIC DOGS AND CATS IN KHARKIV, UKRAINE

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Summary. The spread of dermatophytosis among animals and humans in the localities of Ukraine in particular in the city of Kharkiv is due to the presence of range of the most susceptible animals. This information mainly concerns dogs and cats that can be affected by dermatophytosis and may be a reservoir of dermatophyte fungus. Particularly important is that domestic dogs and cats represent a significant epidemiological threat to the population.

The purpose of the research was to monitor the morbidity of dermatophytosis in domestic dogs and cats in Kharkiv. Diagnostic studies of dermatophytosis morbidity level determination in domestic dogs and cats were performed complexly including the clinical and epizootic data, microscopic and mycological laboratory tests conducted according to generally accepted methods (Kovalenko et al., 2017; Sutton, Fothergill and Rinaldi, 2001).

Dermatophytosis was diagnosed among 231 animals in the study of 1,277 domestic dogs kept by the inhabitants of the city of Kharkiv which was in 18.09% of samples. Dermatophytosis was also detected in 615 animals which constitutes 50.25% in the study of 1,124 cats. The cultures of dermatophytosis agent *Microsporum canis* were isolated from 126 dogs (18.98%) and 110 cats (40.74%). *Trichophyton mentagrophytes* cultures were isolated from 16 dogs (2.41%) and 16 cats (5.93%). The percentage of isolated cultures of mold and yeast-like fungi in the studies of sick dogs and cats were 38.55% and 22.25% respectively. Obtained results indicate quite high level of the dermatophytosis spreading among domestic dogs and cats in the city of Kharkiv.

Keywords: dermatophytosis, domestic animals, dogs, cats

Introduction. Despite significant advances in the study of modern issues of veterinary mycology, dermatophytosis is widespread among animals and humans (Macura, 1993; Shokri and Khosravi, 2016). This is due to the existence of a wide range of animals susceptible to the disease which may be a dermatophyte fungus reservoir (Seker and Dogan, 2011; Kovalenko et al., 2017; Ponomarenko et al., 2018). It concerns small domestic animals, namely dogs and cats, which are kept in their own homes and in the courtyards by inhabitants of large and small settlements of our country including the city of Kharkiv (Ponomarenko, 2017).

Dermatophytoses are the most common mycotic diseases in the world (Kovalenko et al., 2015). These diseases do not cause significant mortality but predetermine a high level of morbidity among animals and humans. Particularly important is that domestic dogs and cats pose a significant epidemiological threat for their owners as well as to other people.

The aim of the study. In a view of above, the goal of our research was to conduct monitoring of dermatophytosis morbidity of domestic dogs and cats in the city of Kharkiv.

Materials and methods. Research on the selected topic, analysis and generalization of obtained data were performed in period from 2012 to 2018 in conditions of private veterinary clinics 'Fauna', 'Aibolit', and 'Avicenna' (Kharkiv) as well as in Educational and Scientific Laboratory of Molecular Genetic Research Methods at the Department of Epizootology and Veterinary Management named after P. I. Verbytskyi in the Kharkiv State Zooveterinary Academy.

Diagnosis of dermatophytosis of domestic dogs and cats was carried out in a complex, taking into account the results of clinical, epizootic, microscopic, and mycological laboratory tests. Animals were tested using Wood's light examination in case of characteristic skin and hair lesions. Biological samples were taken for further laboratory studies after the clinical study.

The study of cultural and morphological properties of isolated cultures of pathogens was carried out using elective nutrient media of Sabouraud agar, Wort Agar, Czapek medium, peptone meat extract glycerol agar, and meat peptone agar. The seedings were incubated in a thermostat at a temperature at a temperature of 28–30 °C for 20–30 days. After that, identification of the isolated cultures of fungus-dermatophytes was carried out using generally accepted methods (Kovalenko et al., 2017; Sutton, Fothergill and Rinaldi, 2001).

Results. During the years 2012–2018, 1,277 dogs and 1,224 cats were examined using clinical-epizootic, microscopic, and mycological methods. Animals with symptoms of skin and hair loss were tested at three private veterinary clinics of the city of Kharkiv. According to research results, dermatophytosis was diagnosed in 846 animals which was 33.83% of the total number of examined animals (Table 1).

Table 1 — Dynamics of dermatophytosis incidence in domestic dogs and cats in the city of Kharkiv

Year of observation	Number of investigated animals, heads		Number of animals with dermatophytosis, heads (%)	
	dogs	cats	dogs	cats
2012	227	215	50 (22.03)	123 (57.21)
2013	217	200	42 (19.35)	111 (55.50)
2014	195	204	38 (19.49)	107 (52.45)
2015	194	181	34 (17.53)	95 (52.49)
2016	193	153	28 (14.51)	67 (43.79)
2017	128	138	24 (18.75)	60 (43.48)
2018	123	133	15 (12.20)	52 (42.28)
Total	1,277	1,224	231 (18.09)	615 (50.25)

The average disease incidence among 1,277 dogs was 18.09% of the number of investigated (231 sick animals) with variation in the number of sick animals from 12.20% in 2018 to 22.03% in 2012.

The average disease incidence among 1,224 cats was 50.25% of the number of investigated (615 sick animals), with fluctuations in the number of diseased animals from 42.28% in 2018 to 57.21% in 2012.

As can be seen from the data presented in Table 1 there is a gradual decrease in the incidence rate both among domestic dogs and domestic cats which are held by the inhabitants of the city of Kharkiv over the period of observation.

The monitoring of pathogens isolated from diseased animals was conducted after determining the level of morbidity of dogs and cats for dermatophytosis. Monitoring studies were carried out in laboratory conditions by studying the cultural and morphological properties of isolated cultures of pathogens on elective nutrient media.

In total, biological material from 664 dogs was investigated. Dermatophytosis was detected in 147 animals which was 22.14% of the number of investigated. In addition, biological material from 270 cats was also studied among which the disease was diagnosed in 126 animals (46.67%).

According to the results of conducted research, cultures of pathogenic dermatophytes, as well as mold and yeast-like fungi with a total of 453 cultures were isolated from domestic dogs and cats (Table 2). Percentage of isolated cultures was calculated from the number of investigated animals of the corresponding species.

Table 2 — Results of monitoring of dermatophytosis pathogens isolated from domestic dogs and cats

Culture	Dogs		Cats	
	Total number	%	Total number	%
Microsporum canis	126	18.98	110	40.74
Trichophyton mentagrophytes	16	2.41	16	5.93
Microsporum gypseum	5	0.75	-	-
Malassezia pachydermatis	64	9.64	18	6.67
Candida albicans	40	6.02	14	5.22
Alternaria alternata	50	7.53	11	4.07
Aspergillus fumigatus	49	7.38	9	3.33
Mucor	53	7.98	8	2.96

According to the data given in Table 2 cultures of *Microsporum canis* were isolated from 126 dogs (18.98%) and 110 cats (40.74%). It indicates a significant advantage of this pathogen in the etiological profile of the disease.

Trichophyton mentagrophytes cultures were isolated from 16 dogs (2.41%) and 16 cats (40.74%). *Microsporum gypseum* cultures were isolated from 5 dogs (0.75%).

The cultures of yeast-like fungi *Malassezia pachydermatis* and *Candida albicans* are isolated in 15.66% of biological samples from dogs and in 11.89% samples from cats. Cultures of mold fungi are isolated in 22.89% of biological material samples from dogs and in 10.36% of samples from cats.

It should be noted that staphylococci, streptococci, and associations of cultures are etiological factors affecting the skin and hair cover of domestic dogs and cats in addition to the aforementioned pathogens (Ponomarenko et al., 2013; Morozova, Severin and Ponomarenko, 2015).

Conclusions. Dermatophytosis was diagnosed among 231 animals which was 18.09% of the number of investigated animals in the study of 1,277 domestic dogs kept by residents of the city of Kharkiv during 2012–2018. Dermatophytosis was detected in 615 animals in the study of 1,124 cats which was 50.25% of the number of investigated ones.

Cultures of dermatophytosis pathogen *Microsporum canis* were isolated from 126 dogs (18.98%) and 110 cats (40.74%). *Trichophyton mentagrophites* cultures were isolated from 16 dogs (2.41%) and 16 cats (5.93%). The share of isolated cultures of mold and yeast-like fungi was 38.55% in the studies of sick dogs and 22.25% for of sick cats.

The data obtained during the research study indicates a rather high spread of dermatophytosis among domestic dogs and cats in the city of Kharkiv.

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Part 2. Biotechnology and genetics

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PCR TEST SYSTEMS FOR THE *CLAVICHLAMYDIA SALMONICOLA* AND *PISCICHLAMYDIA SALMONIS* DETECTION IN FISH

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Summary. The aim of our work was to develop PCR test systems for the identification and differentiation of the *Piscichlamydia salmonis* and *Clavochlamydia salmonicola*, species, that are known epitheliocystis infection agents of gill and fish skin diseases, characterized by the presence of specific 'inclusions' in the epithelial cells of the gills. To date, the diseases of fish associated with chlamydial infections have been detected in more than 90 species of freshwater and marine fish worldwide. For now, there is no available information on the prevalence of *Piscichlamydia salmonis* and *Clavochlamydia salmonicola*, which can cause epitheliocystis of commercially important aquaculture species in Ukraine. Identification of these pathogens is possible only using molecular genetic methods. As a result of our research, we got PCR tests for the identification and species differentiation of *Piscichlamydia salmonis* and *Clavochlamydia salmonicola*. The use of diagnostics for the identification of *Piscichlamydia salmonis* and *Clavochlamydia salmonicola* makes chlamydial infections monitoring among various fish species possible and it will increase the economic efficiency of fish farms.

Keywords: *Piscichlamydia salmonis*, *Clavochlamydia salmonicola*, chlamydial infections in fish, Ukraine, PCR, Atlantic salmon, *Salmo salar*, brown trout, *Salmo trutta*

Introduction. Bacteria of the order Chlamydiales are gram-negative, obligate, and intracellular with a special two-phase development cycle, associated with disease in a wide range of hosts (Ksonz, 2012). More than 400 host species are documented worldwide, most of which are wild animals (Burnard and Polkinghorne, 2016). To date, chlamydial infections have been described in more than 90 freshwater and marine fish species around the world (Pawlikowska-Warych and Deptuła, 2016; Taylor-Brown et al., 2015).

Bacteria of the order Chlamydiales are associated with epitheliocystis, a common condition of the fish gill and fish skin epithelium, characterized by the presence of cytoplasmic bacterial inclusions in the epithelial cells of the gills and on rare occasions spreads to the fish skin (Stride, Polkinghorne and Nowak, 2014; Sellyei, Molnár and Székely, 2017). For the first time, the aforementioned disease has been reported as 'mucophilosis' and was described in the common carp (*Cyprinus carpio*) in 1924 (Plehn, 1924), the term 'epitheliocystis' appeared later, in 1969 (Hoffman et al., 1969).

In most cases epitheliocystis in fish, especially free-living, has a chronic course. This is not a life-threatening disease. Epitheliocystis may cause hypertrophy and inflammation, white nodular lesions of epithelial tissues of gills or skin, gasping at the water surface, lethargy, weak swimming behavior and growth retardation (Lewis et al., 1992). There are known the cases when inflammation extended to other tissues, causing severe respiratory disturbances and death of fish (Meijer et al., 2006; Draghi II et al., 2007). Syasina, Park and Kim (2004) described the case of the disease in a population of fish with a high mortality rate (up to 100%). In addition to clinical manifestations mentioned above, the disease was manifested by exophthalmos, lens lesion, phacocele, corneal clouding and blindness, as well as skin ulcers (Syasina, Park and Kim, 2004).

Initially, it was thought that the same etiologic agent caused epitheliocystis in all fish species, but already in 1977 it was recognized that these bacteria demonstrated a high degree of host specificity (Zachary and Paperna, 1977).

As for *Chlamydia*-like bacteria associated with epitheliocystis, they include:

Candidatus Clavochlamydia salmonicola,
Candidatus Piscichlamydia salmonis,
Candidatus Parilichlamydia carangidicola,
Candidatus Actinochlamydia clariae,
Candidatus Similichlamydia laticola,
Candidatus Similichlamydia labri,
Candidatus Similichlamydia latridicola,
Candidatus Renichlamydia lutjani,
Candidatus Syngnamydia venezia,
and *Neochlamydia*-like bacteria.

Ca. P. salmonis is associated with chlamydial infection in the Atlantic salmon (*Salmo salar*) (Draghi II et al., 2004), and in the brown trout *(Salmo trutta)* (Schmidt-Posthaus et al., 2012). Other family members of Piscichlamydiaceae might be a causative agent of the epitheliocystis of the grass carp (*Ctenopharyngodon idella*) (Kumar et al., 2013), the common carp (*Cyprinus carpio*) and the Gibel carp (*Carassius gibelio*) (Sellyei, Molnár and Székely, 2017).

Ca. C. salmonicola is known to be an intracellular bacteria and infects salmonid fish. For example, the Atlantic salmon (*Salmo salar*) and the brown trout (*Salmo trutta*) are extremely valuable species bred in Ukraine (Schmidt-Posthaus et al., 2012; Guevara Soto et al., 2016; Karlsen et al., 2008; Blandford et al., 2018; Shcherbukha, 1987).

Other *Chlamydia*-like bacteria, such as *Ca.* P. carangidicola, are pathogens of the yellowtail kingfish (*Seriola lalandi)* (Stride et al., 2013a); *Ca.* S. laticola is a pathogen of the Australian barramundi (*Lates calcarifer*) (Stride et al., 2013c); *Ca.* S. labri causes epitheliocystis in the ballan wrasse *(Labrus bergylta),* native inhabitants of the northeastern Atlantic (Steigen et al., 2015)*; Ca.* S. latridicola was isolated from the striped trumpeter (*Latris lineate*), native to the temperate oceans of New Zealand and the eastern coast of Australia (Stride et al., 2013b); *Ca.* A. clariae causes epitheliocystis in the catfish (*Clarias gariepinus*) in Uganda (Steigen et al., 2013); *Ca.* S. venezia is associated with chlamydial infection in the broad nosed pipefish (*Syngnathus typhle*) (Fehr et al., 2013). There are reports of *Neochlamydia*-like bacteria that associated with epitheliocystis in the Arctic charr (*Salvelinus alpinus*) (Draghi II et al., 2007).

Among all these types of chlamydia-like bacteria only *Ca.* C. salmonicola*, Ca. P. salmonis*, and *Ca.* S. venezia can be distinguished in Ukraine, since their owners are common in our country, from which only *Ca.* C. salmonicola and *Ca.* P. salmonis are associated with epitheliocystis infection among the commercially important aquaculture species of Ukraine.

For today, there are no test systems in the arsenal of ichtyopathologists and laboratories of veterinary medicine of Ukraine, which allows detecting, identifying and differentiating *Ca.* C. salmonicola and *Ca.* P. salmonis.

The aim of our work was to develop PCR test systems for the identification and species differentiation of *Ca.* C. salmonicola and *Ca.* P. salmonis, epitheliocystis agents of commercially important aquaculture species of Ukraine.

Materials and methods. The studies were carried out in the Laboratory of animal health and the Laboratory of genetics of the Institute of Pig Breeding and Agroindustrial Production of the National Academy of Agrarian Sciences, which is certified for DNA genetic analyses (Compliance certificate 'state of the measurement system' number 021-19 from 01/31/2019).

Bioinformatics studies were focused on finding the specific region of various types of chlamydia pathogens. Analysis was performed by aligning 111 primary nucleotide sequences of the 16S rRNA gene using the MEGA7 software (Tamura et al., 2007). Species-specific oligonucleotide primers were designed and tested for absence of complementarity with the nucleotide sequences of other microorganisms using the online service Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primerblast/index.cgi).

Molecular genetic analysis was performed using PCR. DNA samples of *Chlamydia*-like organisms pathogenic for fish: *Ca.* C. salmonicola and *Ca.* P. salmonis were used as the positive controls. DNAs were kindly provided by Dr. Heike Schmidt-Posthaus (Center for Fish and Wildlife Health, Bern, Switzerland). The control DNA samples of *Parachlamydia acanthamoebae* strains Berg17 and Bn9 were kindly provided by Dr. Michel Rolf (Central Military Hospital, Koblenz, Germany), DNA of *Parachlamydia acanthamoebae* strain Hall's coccus was received from Prof. Gilbert Greub (Institute of Microbiology of the University of Lausanne, Switzerland), DNA samples of *Waddlia chondrophila, Chlamydia avium, Ch. pecorum, Ch. abortus, Ch. psittaci, Ch. suis, Ch. caviae* were received from Dr. Christiane Schnee (Institute of Molecular Pathogenesis, Jena, Germany). DNA amplification was performed on the 'Tercyc-2' multichannel thermocycler (DNA-Technology LLC, Russia).

Oligonucleotide primers were synthesized (by Metabion International AG): to amplify a fragment of 16S rRNA gene *Ca.* P. salmonis which forward is PICHSF:

CTAGACTAGAGTTCAAGGGGG

and reverse is PICHSR:

GCTAGGGTTGAGACTAGCTAC,

and *Ca.* C. salmonicola forward is CLACHSF:

GAGTTCGTTAAAGCGGGGGA

and reverse is CLACHSR:

CAGGTCTTTCTTGTCCCCAAG.

Amplification was performed according to the manufacturer's protocol (Thermo Fisher Scientific). The identity of the PCR amplification product *of Ca.* C. salmonicola was confirmed by restriction analysis using *Alu* I endonuclease, according to the manufacturer's protocol (Thermo Fisher Scientific).

PCR and restriction products were separated using 2% agarose gel electrophoresis in 1 × TBE buffer for 2 hours at a current of 50 mA in an electrophoresis chamber (Cleaver Scientific Ltd). Plasmids *pUC*19 hydrolyzed with *Msp* I endonuclease (Thermo Fisher Scientific) were used as a molecular weight marker. After the end of the electrophoresis process, the gel was stained with a solution of ethidium bromide (10 mg/cm^3) and the results of

electrophoresis were documented on a transilluminator using a digital camera.

Results of the study. For primers design, the 111 16S rRNA gene primary sequences of 36 species from Chlamydiales order were analyzed. Using MEGA7 software, alignment of primary sequences obtained from the international databases GenBank and NCBI was carried out, as a result of which the common nucleotide sequence segments were found for both bacteria, and also specific regions of the nucleotide sequences of *Ca.* C. salmonicola and *Ca.* P. salmonis were found.

Amplification of control *DNA*s of *Ca.* C. salmonicola and *Ca.* P. salmonis with corresponding primers and the following gel electrophoresis of PCR products determined the size of the amplified DNA fragments as 207 base pairs (bp) and 276 bp respectively, the fragments corresponded to the expected sizes of the DNA fragments of 16S rRNA gene of *Ca.* C. salmonicola and *Ca.* P. salmonis (Fig. 1).

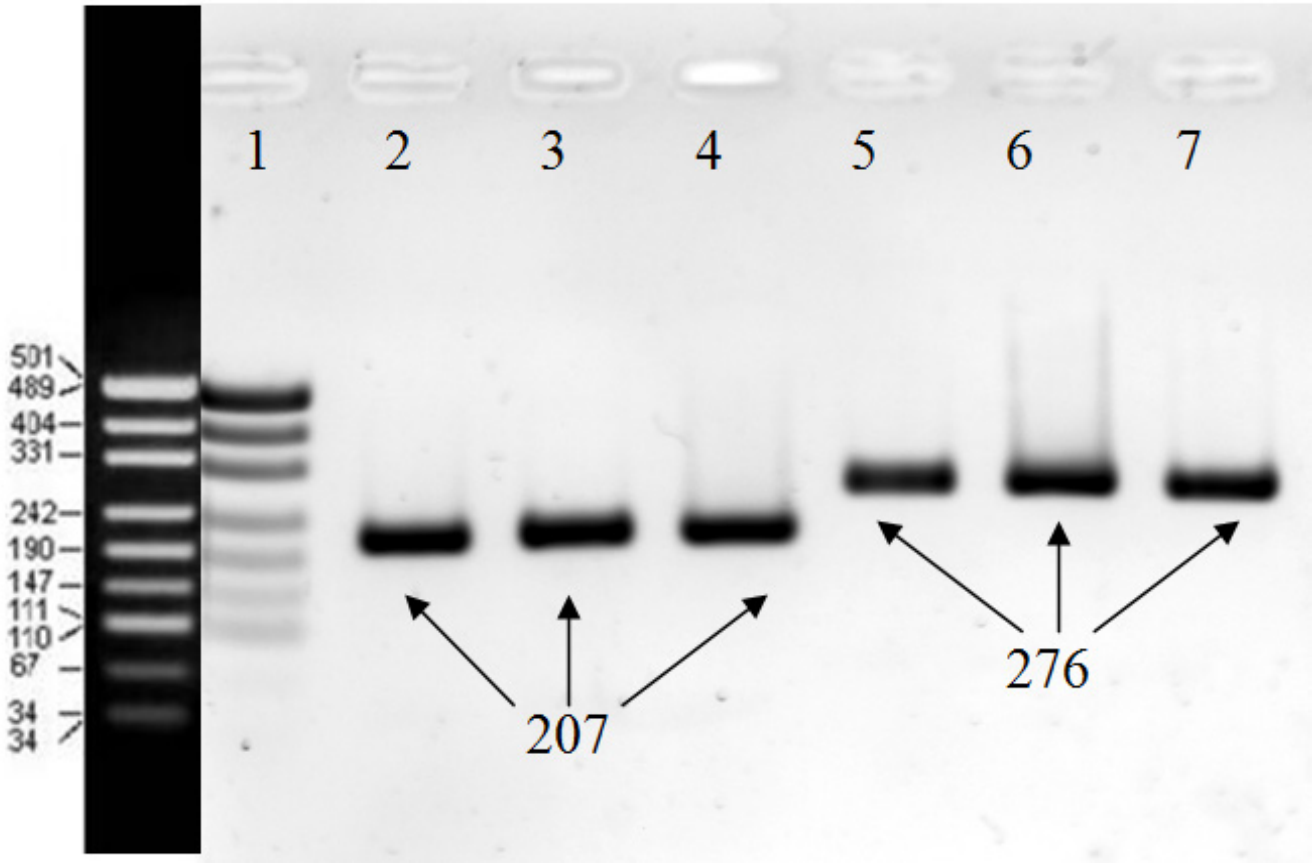

Figure 1. Electrophoregram of *Ca.* Piscichlamydia salmonis and *Ca.* Clavochlamydia salmonicola control DNAs amplification with PICHSF/PICHSR and CLACHSF/CLACHSR primers, using different annealing temperature: 1 — DNA size marker *pUc*19/*Msp*I; 2–4 — PCR detection of *Ca.* Piscichlamydia salmonis 58 °C, 60 °C, 63 °C respectively; 5–7 — PCR detection of *Ca.* Clavochlamydia salmonicola 58 °C, 60 °C, 63 °C respectively.

The restriction analysis with *Alu* I endonuclease demonstrated the formation of three DNA fragments of 61 bp, 19 bp, and 196 bp, corresponding to the expected with the primary nucleotide sequence of *Ca.* C. salmonicola (Fig. 2a). To verify the identity of the PCR product, for *Ca.* P. salmonis identification, was used a *Tas* I endonuclease that formed two fragments of 29 bp and 178 bp (Fig. 2b.).

Thus, the identity of the PCR amplification product has been proven. The verification of the PCR test analytical specificity performed by amplifying the positive control DNAs of 10 species from Chlamydiales order showed the absence of PCR products, but expected one (Fig. 4, 5).

Discussion. The diagnosis of epitheliocystis in fish is mainly based on the clinical signs evaluation, including visible damage to the gills and skin, changes in the behavior of the fish. It is only possible to make a preliminary diagnosis of epitheliocystis based on morphological changes that can be detected by microscopy, but the identification of the pathogen is possible only by using molecular genetic diagnostic methods (Blandford et al., 2018; Pawlikowska-Warych and Deptuła, 2016).

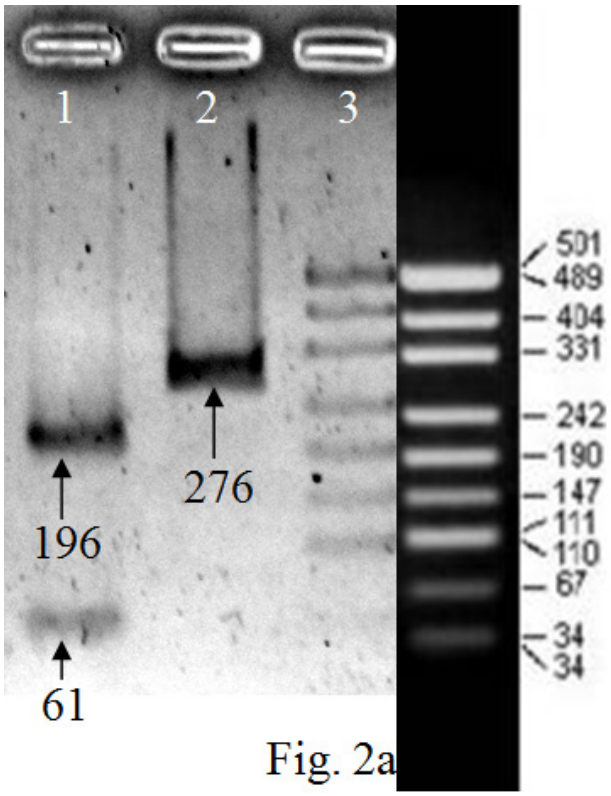

Figure 2a. Electrophoregram of *Ca.* Clavochlamydia salmonicola control DNA amplification with CLACHSF/CLACHSR primers and restriction of PCR product with *Alu* I endonuclease: 1 — restriction fragments of *Ca.* Clavochlamydia salmonicola PCR products (196 bp and 61 bp); 2 — absence of *Ca.* Clavochlamydia salmonicola DNA restricted PCR product (276 bp); 3 — DNA size marker *pUc*19 /*Msp*I.

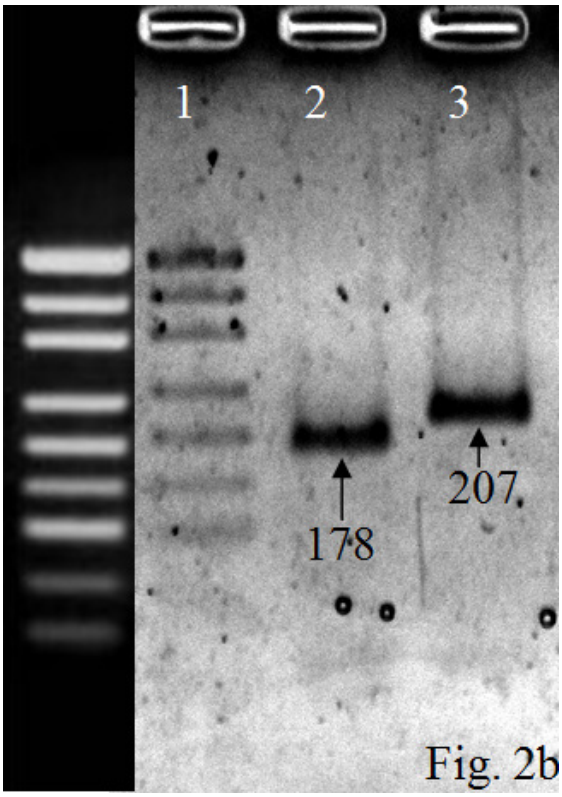

Figure 2b. Electrophoregram of *Ca.* Piscichlamydia salmonis control DNA amplification with PICHSF/PICHSR primers and restriction analysis with *Tas* I endonuclease: 1 — marker for DNA size *pUc*19/*Msp*I; 2 — restriction fragments of *Ca.* Piscichlamydia salmonis PCR product (178 bp and 29 bp are not shown in the picture); 3 — absence of *Ca.* Piscichlamydia salmonis DNA restricted PCR product (207 bp).

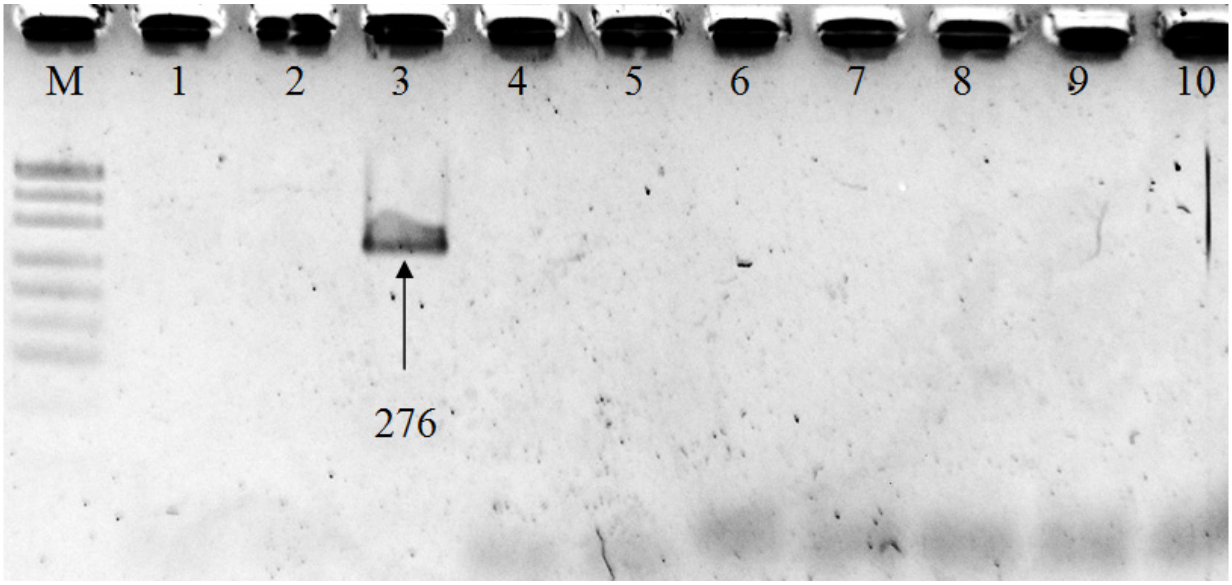

Figure 4. Electrophoregram of PCR products with primers CLACHSF/CLACHSR for *Ca.* Clavochlamydia salmonicola detection and DNA of 9 Chlamydiales species: M — DNA size marker *pUc*19/*Msp*I; 1 — *Parachlamydia acanthamoebae*; 2 — *Waddlia chondrophila*; 3 — *Ca.* Clavochlamydia salmonicola (267 bp); 4 — *Ca.* Piscichlamydia salmonis; 5 — *Chlamydia avium*; 6 — *Chlamydia pecorum*; 7 — *Chlamydia abortus*; 8 — *Chlamydia psittaci*; 9 — *Chlamydia suis*; 10 — *Chlamydia caviae.*

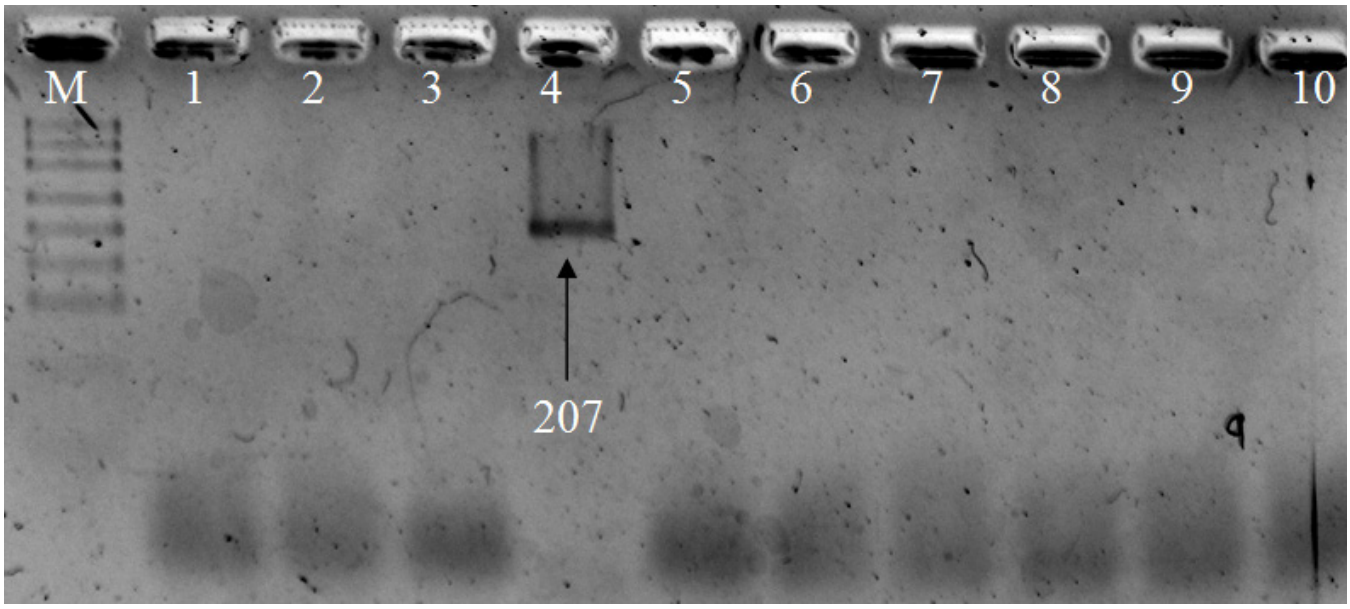

Figure 5. Electrophoregram of PCR products with primers PICHSF/PICHSR for *Ca.* Piscichlamydia salmonis detection and DNA of 9 Chlamydiales species: M — DNA size marker *pUc*19/*Msp*I; 1 — *Parachlamydia acanthamoebae*; 2 — *Waddlia chondrophila*; 3 — *Ca.* Clavochlamydia salmonicola; 4 — *Ca.* Piscichlamydia salmonis (207 bp); 5 — *Chlamydia avium*; 6 — *Chlamydia pecorum*; 7 — *Chlamydia abortus*; 8 — *Chlamydia psittaci*; 9 — *Chlamydia suis*; 10 — *Chlamydia caviae.*

Developed PCR tests for the identification and differentiation of *Ca.* C. salmonicola and *Ca.* P. salmonis have shown their suitability for amplifying positive control DNA. A restriction analysis with *Alu* I endonuclease enzyme confirmed that the amplification was performed on the DNA fragment of the 16S rRNA gene of the *Clavochlamydia salmonicola*.

There are foreign-made commercial PCR tests to identify *Ca.* C. salmonicola and *Ca.* P. salmonis, which are not available for mass use in Ukraine in the current economic situation, due to the high price and/or necessity of expensive equipment (especially, for qPCR providing). Thus, simple, practical, inexpensive methods were chosen for made it possible to put into practice the diagnosis of these bacterial infections.

PCR tests were also developed to identify and differentiate *Ca.* C. salmonicola and *Ca.* P. salmonis, they demonstrated high analytical specificity — lack of amplification (expected only one case) with the control DNA matrix of 10 species from Chlamydiales *(P. acanthamoebae*, *W. chondrophila*, *Ca.* C. salmonicola, *Ca.* P. salmonis, *Ch. avium*, *Ch. pecorum*, *Ch. abortus*, *Ch. psittaci, Ch. suis*, *Ch. caviae)*.

As a result, PCR tests for identification and species differentiation of *Ca.* C. salmonicola and *Ca.* P. salmonis can be recommended for clinical trials in Ukraine.

Conclusions. 1. As a result of our research, PCR tests for the identification and species differentiation of *Ca.* C. salmonicola and *Ca.* P. salmonis were developed.

2. The use of diagnostic kits for *Ca.* C. salmonicola and *Ca.* P. salmonis identification will help to increase the economic efficiency of fisheries and will allow to provide the monitoring of chlamydial infection among various fish species.

Perspectives for future research. The developed PCR tests for identification and species differentiation of *Ca.* C. salmonicola and *Ca.* P. salmonis after trials on clinical material can be used by scientists for extensive monitoring of epitheliocystis, veterinary medicine doctors to clarify the diagnosis, as well as introduced into the practice of veterinary medicine laboratories and used in fish farms improvement programs.

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Part 3. Biosafety

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ACUTE AND SUB-ACUTE TOXICITY ASSESSMENT OF 'RYBOZURIL' ON COMMON CARP

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Summary. The goal of the work was to study of acute and sub-acute toxicity parameters of 'Rybozuril' biological product (AI — diclazuril) on the model of carp. This drug is effective in the treatment of diseases caused by parasitic Eimeriidae. Carp scales of two years old were used in experiments. To determine acute toxicity, the fish were prescribed with diclazuril in doses of 1 g/kg, 5, 10, and 15 g/kg of live weight. Two experimental and control fish groups of 30 individuals each were formed to determine sub-acute toxicity of 'Rybozuril'. Experimental groups of fish were prescribed with 'Rybozuril' in a dose (by AI) of 50 and 10 mg/kg for two consecutive days. Blood samples were collected from six fish from each group for clinical and biochemical indicators after 2, 7, 14, 21, and 28 days. The hemoglobin content, number of red blood cells and leukocytes blood was determined. The intensity of peroxide oxidation of lipids (POL), catalase activity, level of total antioxidant capacity (TAC), total proteins, albumin, globulins and glucose, circulating immune complexes (CIC) and seromucoids concentration, level of enzymatic activity: aspartate transaminase (AST), alanine transaminase (ALT), α-amylase blood plasma were determined.

The acute toxicity of diclazuril for carp was estimated, LD_{50} is more than 15,000 mg/kg of live weight, the toxicity of diclazuril can be classified as undifferentiated and, in terms of toxicity, it can be classified as hazard class IV. Two administrations of the drug 'Rybozuril' in a daily dose of 50.0 mg/kg of live weight, the maximum expression of metabolic changes in fish was detected from the initial terms of the studies and up to day 21. According to the results the toxic effect of the drug in fish was estimated, which did not influence to a number of indicators. The drug in such dose was shown immunosuppression and membrane-toxic effects in fish. Two-time administration of the 'Rybozuril' drug in a daily dose of 10.0 mg/kg body weight leads to metabolic alterations in fish due to the activation of detoxification processes and lipoperoxidation maintenance in cell membranes at the physiological level. At the end of the experiment the toxic effect of 'Rybozuril' in fish characterized by stable parameters in comparing to the control group.

Keywords: diclazuril, fish, lethal dose, acute toxicity, submerged toxicity, blood, clinical and biochemical indicators

Introduction. Diclazuril ($C_{17}H_9Cl_3N_4O_2$) is a benzeneacetonitrile derivative. Up to now the mechanism of action has not been well studied. It effectively prevents early developmental stages of the Protozoa. Diclazuril is intended for use in the control of eimeriosis in poultry and mammalian livestock. According to the previous studies, diclazuril is a low toxic agent. Thus, oral or subcutaneous administration of diclazuril in a dose of 5,000 mg/kg does not cause death in mice and rats. The administration of diclazuril in rats at a dose of 80 mg/kg of weight for three months did not lead to significant changes in morphological and biochemical blood parameters (Conway et al., 2001; Verheyen et al., 1988; FEEDAP, 2014, 2015; EMEA, 1996; Tokarev et al., 2012; Selifanova and Birukova, 2012).

Diclazuril is highly effective against fish diseases caused by parasitic protozoa from the family Eimeriidae of Apicomplexa (Molnár and Ostoros, 2007). The easiest families of Eimeriidae are widespread among fish inland waters of Ukraine. In the conditions of natural hydroecosystems *Goussia cyprinorum*, *G. castravetsi*, *G. metschnikowi*, *G. alburni*, *G. carpelli*, *G. leucisci*, *G. syngnathi*, *Eimeria scardini*, *E. rutili*, and *E. radae* are registered. For industrial species of pond farms of particular importance are the species *Goussia carpelli*, *G. subepithelialis*, *G. sinensis*, *G. cheni*. Tilapia is infected by *Goussia cochlidarum* in the closed water supply systems (Maltsev, 2012; Trombitskiy and Bushuev, 2012).

The 'Rybozuril' drug (contains 10% of the active substance diclazuril), according to the results of previous studies, proved to be highly effective in controlling fish diseases caused by Eimeriidae protozoa families. Therefore, determination of the impact of this drug is an important step for further studies.

The aim of the study was to determine the parameters of acute (lethal) and sub-acute toxicity of the drug 'Rybozuril' using common carp (*Cyprinus carpio*) as a model.

Materials and methods. A study on the acute toxicity of diclazuril was carried out as described (Kotsiumbas et al., 2006) modified for carp. Two-year-old carp was used for the experiments.

A preliminary study was conducted to determine the approximate average lethal dose. The drug was administrated individually through a catheter in a dose of 1, 5, 10, and 15 g/kg of body weight based on 1% starch paste.

The effect of the 'Rybozuril' drug on the biochemical and clinical parameters of fish blood was determined in the dynamics of the sub-acute toxicological experiment. The experiment was conducted using a two-year-old carp. Two experimental and one control groups of 30 fishes each were formed. Fish of each group were kept in separate aquariums with capacity of 0.2 m^3 with artificial aeration and temperatures of 18–22 °C. In the first and second experimental groups, fish for two consecutive days prescribed the 'Rybozuril' in a daily dose of 50 and 10 mg/kg body weight (for AI) respectively. The drug was prescribed to fish individually using a catheter based on 1% starch paste. In the control group, a starchy paste was added without the preparation (Demidov and Berezkina, 1986).

For the purpose of clinical and biochemical studies blood sampling was carried out from six fish from each group on 2^{nd}, 7^{th}, 14^{th}, 21^{st}, and 28^{th} days. Blood samples was collected by a paste-like pipette from the tail artery according to the generally method. Obtained blood samples were stabilized with heparin, to determine the biochemical parameters of blood serum.

Fish manipulation was carried out in accordance with existing regulations governing the organization of work using experimental animals and observance of the principles from European convention for the protection of vertebrate animals used for experimental and other scientific purposes (CEC, 1986).

The intensity of peroxide oxidation processes in blood plasma was determined by the level of production of its products: primary conjugated dienes (CD) and malonic dialdehyde (MDA) under extraction conditions in a mixture of heptane isopropanol (1:1) as previously described (Gavrilov and Mishkorudnaya, 1983); at wavelengths of 233 and 247 nm; the values of CD expressed as μmol/dm^3, and MDA at specific absorption rate in 1.0 cm^3 ($\Delta D/cm^3$).

The catalase activity in blood plasma was determined, as described (Korolyuk et al., 1988), using H_2O_2 with incubation medium (0.04412 N solution of H_2O_2, 0.01 N solution of KH_2PO_4, 0.1 M Tris-HCl buffer, pH 7.4, 4.5% solution of ammonium molybdenum acid); at a temperature of 37 ± 1 °C; at a wavelength of 410 nm; expressed as μmol H_2O_2/dm^3 in 1 min.

The measurement of antioxidant activity (TOL) in blood plasma was carried out in accordance with (Klebanov et al., 1988), by the total ability of structural antioxidants to inhibit the accumulation of TBA-active products induced in a medium of 25 mM $FeSO_4$ in 0.002 N HCl; at a wavelength of 535 nm; expressed as % of the formation inhibition of TBA-active products.

The level of hematological parameters and the content of total proteins, albumin, glucose and the level of enzymatic activity: aspartate aminotransferases (AST), alanine aminotransferases (ALT), α-amylase in blood plasma were determined by generally accepted methods and using Cormay (Poland) reagent kits as described in the manual (Vlizlo, 2012). The concentration of circulating immune complexes (CIC) of the average molecular weight was determined, as described (Kondrakhin et al., 1985), by precipitation of the protein complexes antigen-antibody PEG-6000; serum cords as described (Men'shikov, 1987); at wavelength of 260 and 280 nm; expressed as mg/cm^3. Registration of biochemical parameters was carried out using a spectrophotometer Shimadzu UV-1800 (Japan).

Statistical analysis of the obtained results was carried out in accordance with the recommendations on biometrics using the Microsoft Excel for Windows XP application package.

Results and discussion. At the determining of the acute lethal dose for pond fish based on the previous experiments for estimation of limit lethal dose of diclazuril we did not observe fish death during 21 days. The administration of diclazuril in a dose of more than 15 g/kg body weight was not feasible, considering that the maximum concentration of the drug that can be created in the starch gel is 25% which is physiologically impossible to administrate starch paste in a volume of more than 60 ml/kg of weight.

Due to the absence of fish death throughout the experiment, the median lethal dose LD_{50} could not determine. At the 21^{st} day after treatment, the fishes were euthanized with the further necropsy, which showed no special changes in the internal organs.

Consequently, due to the absence of fish death, LD_{50} was not able to estimate when the maximum dose of diclazuril, but according to the results of the study, it is greater than 15 g/kg weight. In accordance with the classification proposed by Medved', Kagan and Spynu (1968) and adopted by the WHO, the toxicity of the test drug can be classified as non-expressed, and regarding the state standard (GOST 12.1.007-76) diclazuril can be classified as toxicity class IV.

Clinical and biochemical parameters of blood after administering a therapeutic dose and five times higher therapeutic dose were analyzed to determine the effect of the drug 'Rybozuril' on fish. During the experiments behavioral changes of fishes in the experimental and control groups were not observed.

Table 1 shows the dynamics of hematological parameters in each group.

The data present in Table 1 indicates no statistical changes in the hematological parameters for both doses.

Table 2 shows the results of determining the basic biochemical parameters of blood and markers of congenital immunity of fish during 'Rybozuril' exposure.

Table 1 — Hematological parameters in carp after two administrations of 'Rybozuril' in a daily dose of 50.0 and 10 mg/kg body weight (n = 6; M ± m)

Parameters	Groups	Period of experiment, day				
		2	7	14	21	28
Hemoglobin, g/l	I	98.3 ± 1.8	96.8 ± 4.3	96.6 ± 1.7	98.8 ± 1.8	101.3 ± 1.4
	II	98.4 ± 2.0	93.2 ± 1.0	97.5 ± 3.9	98.8 ± 1,5	98.6 ± 3.1
	Control	99.5 ± 3.7	92.2 ± 1.5	96.3 ± 5.1	100.2 ± 2.7	101.3 ± 0.9
Red blood cells, 10^{12}/l	I	7.6 ± 0.4	7.7 ± 0.3	7.9 ± 0.1	7,6 ± 0,1	7.6 ± 0.2
	II	7.8 ± 0.5	8.2 ± 0.3	8.2 ± 0.3	7.9 ± 0,3	7.8 ± 0.1
	Control	7.6 ± 0.2	7.8 ± 0.4	7.6 ± 0.1	7.8 ± 0.1	7.8 ± 0.3
White blood cells, 10^9/l	I	19.2 ± 0.2	19.2 ± 0.4	18.9 ± 0.2	19.5 ± 1.1	19.2 ± 0.7
	II	20.1 ± 0.7	19.5 ± 0.3	19.3 ± 04	19.2 ± 0.9	19.3 ± 0.7
	Control	19.6 ± 0.6	19.6 ± 0.5	19.8 ± 0.9	19.8 ± 0.6	19.9 ± 0.6

Notes: * — p ≤ 0.1, ** — p ≤ 0.05, *** — p ≤ 0.01, **** — p ≤ 0.001.

Table 2 — Biochemical parameters in carp after two administrations of 'Rybozuril' in a daily dose of 50.0 and 10 mg/kg body weight (n = 6; M ± m)

Parameters	Group	Period of experiment, day				
		2	7	14	21	28
Total protein, g/l	I	32.86 ± 1.08	32.60 ± 0.56	32.24 ± 1.60	32.42 ± 0.68	32.60 ± 1.37
	II	32.69 ± 1.02	31.96 ± 0.66	31.82 ± 0.76	31.92 ± 1.14	33.02 ± 1.03
	Control	33.42 ± 1.0	34.21 ± 1.01	33.81 ± 1.14	33.62 ± 1.05	32.62 ± 0.76
Albumins, g/l	I	19.38 ± 0.39	18.41 ± 0.59	18.96 ± 0.97	19.18 ± 0.68	19.59 ± 1.20
	II	19.66 ± 0.22	18.74 ± 0.15	19.04 ± 0.49	18.66 ± 0.56	19.74 ± 0.29
	Control	19.60 ± 0.30	20.12 ± 1.28	20.90 ± 1.03	19.83 ± 0.78	19.64 ± 1.04
Globulins, g/l	I	13.48 ± 1.04	14.20 ± 0.93	13.28 ± 1.72	13.24 ± 0.93	13.02 ± 2.14
	II	13.03 ± 1.14	13.22 ± 0.80	12.79 ± 0.71	13.26 ± 1.18	13.28 ± 1.0
	Control	13.82 ± 0.71	14.10 ± 1.88	12.91 ± 0.66	13.79 ± 0.66	12.98 ± 0.84
CIC, mg/ml	I	0.13 ± 0.005**	0.115 ± 0.012	0.110 ± 0.004	0.118 ± 0.003	0.115 ± 0.006
	II	0.105 ± 0.003	0.113 ± 0.005	0.110 ± 0.004	0.115 ± 0.003	0.118 ± 0.009
	Control	0.110 ± 0.004	0.118 ± 0.009	0.120 ± 0.004	0.120 ± 0.004	0.118 ± 0.007
Sm, mg/ml	I	0.29 ± 0.01****	0.28 ± 0.01****	0,26 ± 0.01****	0.230 ± 0.004***	0.20 ± 0.01
	II	0.18 ± 0.01	0.20 ± 0.01	0,178 ± 0,009	0,20 ± 0,004	0,20 ± 0,004
	Control	0.17 ± 0.01	0.18 ± 0.01	0.178 ± 0.005	0.19 ± 0.01	0.193 ± 0.003

Notes: * — p ≤ 0.1, ** — p ≤ 0.05, *** — p ≤ 0.01, **** — p ≤ 0.001.

It was found that in blood plasma of fish from experimental groups I and II, there was no statistical changes in the content of total proteins during the experiment. An exception was the tendency to decrease its level on 21st day after the drug administration. However, during the introduction of the drug in both doses on the 7th and 14th day of the experiment, a tendency to decrease their albumin fraction was determined, which for the fish from group I and II averaged at 8.9 and 7.2% respectively. At the end of the experiment, the value of the indicator did not acquire statistical changes and close to their control level.

In addition, the toxic manifestation of the action of the drug at a higher dose (experiment I) indicates an increase of the level of the formation of osmotic phase proteins — seromucoids, the maximum content of which in the blood plasma of fish was set on the 2nd day after the introduction of 'Rybozuril' (70.6%, $p < 0.05$). On the 7th, 14th, and 21st day of the experiment, an increase in serum levels in the blood plasma of fish and experimental group was 55.6, 44.4, and 21.1% ($p < 0.05$) respectively, relative to the control values of the indicator. The obtained results are consistent with the increase of the level of circulating immune complexes (CIC) of the average molecular weight in blood plasma of fish in this group on the 2nd day after administration of the drug, which is an average of 16.3% ($p < 0.05$) relative to control.

The CIC are constantly circulating in the blood, which are important immune homeostasis components, and their elimination occurs by the cells of the reticuloendothelial system (Levinsky, 1981). It should be noted that the probable CIC increasing the physiological

products of the reaction 'antigen'-'antibody', which was determined by the effect of the drug in a toxic dose (experiment I), might be explained either by the antibodies development due to dysfunction of mechanisms of nonspecific protection in the forced mode, or by a shift in the elimination of CIC due to the functioning of the reticuloendothelial system (Vlizlo, 2012).

This fact, along with the increasing in the formation of serum counts in fish from this experimental group can reflect the phases of the development of immunotoxic reactions in their organisms.

The protein metabolism activity, as well as the enhancement of the formation of lipoproteins, which are necessary for the restoration of affected cell membranes, is shown by the action of a number of xenobiotics (Al-Akel et al., 2010).

The intensity of the processes of lipoperoxidation and the activity of the antioxidant system in carp by using the 'Rybozuril' drug is shown in Table 3.

Table 3 — The intensity of lipoperoxidation processes and the activity of the antioxidant system in carp after two administrations of 'Rybozuril' in a daily dose of 50.0 and 10 mg/kg body weight (n = 6; M ± m)

Parameters	Group	Period of experiment, day				
		2	7	14	21	28
Peroxide oxidation of lipids (POL)						
Conjugated dienes, μmol/l	I	15.60 ± 0.87****	22.41 ± 0.65*	27.86 ± 1.63	27.22 ± 1.66	25.04 ± 0.47
	II	25.33 ± 0.95	24.98 ± 1.15	25.42 ± 1.57	28.97 ± 1.48	25.50 ± 2.08
	Control	26.02 ± 1.51	25.85 ± 1.67	26.15 ± 0.42	26.31 ±0.28	25.96 ± 2.08
Malonic dialdehyde, $\Delta D/cm^3$	I	3.38 ± 0.37**	4.08 ± 0.47**	4.40 ± 0.28**	4.44 ± 0.37**	4.54 ± 0.28**
	II	5.44 ± 0.50	5.75 ± 0.11	5.85 ± 0.70	5.40 ± 0.50	6.10 ± 0.17
	Control	5.64 ± 0.28	5.74 ± 0.27	5.74 ± 0.38	5.80 ± 0.16	5.81 ± 0.41
Level of total antioxidant capacity (TAC)						
Catalase activity, μmol H_2O_2/dm^3 in 1 min	I	21.6 ± 0.3*	20.9 ± 0.5	20.8 ± 1.0	20.1 ± 0.8	19.3 ± 0.3
	II	26.9 ± 1.5***	28.0 ± 0.6****	30.4 ± 3.0**	26.2 ± 1.4***	22.3 ± 0.6*
	Control	19.6 ± 0.7	19.6 ± 0.6	20.1 ± 0.3	19.9 ± 0.6	20.2 ± 0.7
Inhibition, %	I	58.0 ± 4.9	59.2 ± 2.7	58.4 ± 1.8	58.9 ± 2.6	58.6 ± 2.3
	II	52.2 ± 9.8	53.1 ± 4.4	47.3 ± 3.4**	43.0 ± 5.8**	35.5 ± 2.6****
	Control	57.7 ± 4.6	58.6 ± 6.3	58.7 ± 1.1	57.3 ± 0.7	59.1 ± 1.6

Notes: * — $p \le 0.1$, ** — $p \le 0.05$, *** — $p \le 0.01$, **** — $p \le 0.001$.

The membrane-toxic effect of the drug at a higher dose indicates inhibition of the intensity of the processes of peroxide oxidation of lipids by the level of formation of lipoperoxidation products in fishes and experimental group (Table 3). Thus, on the 2nd and 7th day of the experiment, blood plasma of fish in this group recorded decrease in the level of primary lipid oxidation products (LOPs) — conjugated dienes an average of 40.0 and 13.3% ($p < 0.05$) relative to control. The value of the indicator, from the 14th day and at the end of the experiment (after 28 days), reached its control level.

However, from the 2nd to 28th day of the experiment, in the blood plasma of fish from group I, a decreasing of terminal membrane-toxic products of lipoperoxidation — MDA formation was determined. Thus, on 2nd, 7th, 14th, 21st, and 28th day after drug administration at a higher dose, the reduction of the MDA content in the plasma of fish was 40.0, 29.0, 23.3, 23.4, and 21.9% ($p < 0.05$) respectively, in relation to the control values.

The total antioxidant capacity (TAC) in blood plasma of fish at the effect of the drug in a lower dose had other changes. Thus, fishes of the group II during the experiment, an increase in the enzymatic activity of catalase was detected on average by 34.7% ($p < 0.05$) in comparison to the control. In the same time in the blood samples from fishes in this group, a gradual consumption of non-enzymatic levels of TAC was found on the dynamics of the overall TAC index. Thus, on 2nd, 7th, 14th, 21st, and 28th day after the introduction of the drug, the level of this indicator decreased in blood plasma of fish in comparison to its control values by 9.5, 9.4, 19.4, 25.0, and 40.0% ($p < 0.05$) respectively.

Therefore, it should be noted that during the experiment in the fish which obtained drug at a lower dose (group II), the intensity of the processes of the POL (according to the level of formation of its primary and end products) was at the level of physiological control. It could be explained by occurring of compensatory expenditure of a pool of endogenous structural antioxidants (at TAC decreasing) and adaptogenic enhancement of catalase activity: the maximum expressed changes in the values of the parameters were found on the 28th and 14th day of the experiment. It is known that catalase plays a major role in the adaptation of cells to the increased intensity of catabolic and destruction (Marques et al., 2015), and the determined dynamics of this enzyme as a result of the

introduction of the drug is cells adaptation to maintain the processes of lipoperoxidation in their membranes at the physiological level.

The dynamics of enzymes and glucose in the blood plasma of carp for the use of drug 'Rybozuril' is shown in Table 4.

Table 4 — Enzymes and glucose dynamics in blood plasma of carp after two administrations of 'Rybozuril' in a daily dose of 50.0 and 10 mg/kg body weight (n = 6; M ± m)

Parameters	Groups	Period of experiment, day				
		2	7	14	21	28
ALT, mmol/(h×l)	I	0.37 ± 0.01	0.37 ± 0.02	0.4 ±0.01	0.38 ±0.01	0.4 ±0.01
	II	0.32 ± 0.01***	0.3 ± 0.01**	0.38 ±0.02	0.44 ±0.03	0.4 ±0.03
	Control	0.36 ± 0.003	0.37 ±0.02	0.38 ±0.01	0.40 ±0.04	0.41 ±0.01
AST, mmol/(h×l)	I	1.98 ± 0.03	2.12 ±0.03	1.59 ± 0.04**	1.77 ± 0.05**	1.58 ± 0.05***
	II	2.61 ± 0.02**	1.8 ± 0.03***	1.59 ± 0.05**	2.15 ±0.04	2.17 ±0.06
	Control	2.13 ± 0.12	2.1 ±0.06	2.07 ±0.11	2.11 ±0.10	2.09 ± 0.09
α-amylase, mg/(sec×l)	I	5.10 ± 0.13	4.61 ± 0.19	4.44 ± 0.03****	4.48 ± 0.05****	5.07 ± 0.05
	II	5.07 ± 0.08	5.11 ± 0.09	5.25 ± 0.14	5.29 ± 0.13	5.10 ± 0.04
	Control	4.98 ± 0.03	4.97 ± 0.03	4.97 ± 0.03	5.01 ± 0.02	5.08 ± 0.07
Glucose, mmol/l	I	3.52 ± 0.04	3.17 ± 0.05***	3.04 ± 0.09**	3.51 ± 0.16	3.53 ± 0.13
	II	3.48 ± 0.09	3.38 ± 0.06	3.35 ± 0.07	3.45 ± 0.03	3.6 ±0.05
	Control	3.56 ± 0.06	3.51 ±0.03	3.52 ±0.14	3.57 ±0.14	3.56 ±0.13

Notes: * — $p \le 0.1$, ** — $p \le 0.05$, *** — $p \le 0.01$, **** — $p \le 0.001$.

It was noticed that there is decrease the level of glucose in the blood of fish from group I on 7th and 14th day of the experiment, having valued 9.7 and 13.6% ($p < 0.05$) respectively in comparison to the control. The results suggested compensatory character and aimed at activation of detoxification processes due to administration of the drug in a toxic dose. At the end of the experiment (on the 21st and 28th day), the value of the indicator was not statistically different from the control.

It is known that the magnitude of the induction of activity of hepatic enzymes in the blood is proportional to the degree of destruction of hepatocytes and the activity of the pathological process (Khazanov, 1988, De Bono, 1994).

The drug administration at a lower dose at the beginning of the experiment affected the intensity of the transamination processes in the liver and myocardium of fish (Table 2). Thus, on the 2nd and 7th day of the experiment in blood plasma of fish from group II, a significant decrease in the enzymatic activity of ALT was recorded on average by 11.1 and 18.9% ($p < 0.05$) relative to the control.

Whereas, on the 2nd day, the AST level, which participates in providing cytoplasm to substrates for gluconeogenesis in the conversion of pyruvate to glucose (Dirksen, 1990), in the blood of fish from this group significantly increased by 22.5%, and on the 7th day decreased by 14.3% relative to its control values. Thus, the obtained results indicate, on the one hand, the temporal inhibition of the processes of transamination, and on the other hand there is intensity of detoxification systems in the liver of experimental fish.

Glucose decreasing in blood plasma of fish was observed on the 7th and 14th day as a result of drug administration in a toxic dose. It suggests the participation of α-amylase, the activity of which at this time was reduced by 7.2 and 10.7% respectively relative to the control level of the enzyme. The established changes in the indicators at the end of the experiment had a restoration to the physiological values.

Thus, the maximum severity of metabolic changes in fish after two-time administration of the 'Rybozuril' drug in a daily dose of 50.0 mg/kg body weight was found at the initial stages of the study and until 21st day. According to the obtained results, manifestations of the toxic effect of the drug in fish, that had no reciprocal character by a number of indicators. The drug in such dose was shown immunosuppression and membrane-toxic effects in fish.

The metabolic alterations in fish due to the drug effect at a lower dose (group II) influence on the activation of detoxification processes and maintenance of processes of lipoperoxidation in cell membranes at the physiological level. It was stated that at the end of the experiment dynamics of most of the investigated parameters had a reversible character and by their value approached the physiological level (control).

Conclusions. 1. Due to the absence of fish death after determining the maximum dose of diclazuril LD_{50} it was not possible to determine. However, it can be classified as hazard class IV toxicity.

2. Two-time administration of the 'Rybozuril' drug in a daily dose of 50.0 mg/kg body weight, the maximum exposure to metabolic changes in fish was observed in the initial terms of the study until 21st day. Based on the

obtained results, manifestations of the toxic effect of the drug in fish at the end of the experiment dynamics of most of the investigated parameters had not a reversible character. The drug in such dose was shown immunosuppression and membrane-toxic effects in fish.

3. Two-time administration of the 'Rybozuril' drug in a daily dose of 10.0 mg/kg body weight leads to metabolic alterations in fish due to the activation of detoxification processes and lipoperoxidation maintenance in cell membranes at the physiological level. At the end of the experiment the toxic effect of 'Rybozuril' in fish characterized by stable parameters in comparing to the control group.

Outlooks. Taking into account the obtained results and data of previous studies, it is planned to introduce the 'Rybozuril' drug into the practice of veterinary medicine and fish farming in order to control fish diseases caused by the parasitic protozoa from family Eimeriidae.

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