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.

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

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INFRARED MILK PASTERIZER AS A COMPONENT OF SUCCESS IN THE ANIMAL LEUKEMIA CONTROL

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Summary. One of the most common and dangerous cattle diseases of oncogenic origin is leukemia. An effective technological step to control animal leukemia and to prevent the possibility of its further spread is milk pasteurization. We have studied the quality of dairy raw materials and equipment used in the pasteurization of milk. The resistance of pasteurized milk was compared after using various methods of its processing (storage in a refrigerator at a temperature of 4–5°C). The comparative characteristics and specific energy consumption of the most popular pasteurizer models with 'UOM' milk pasteurizer-disinfectant were described. We studied the specific energy consumption of the 'UOM' units. It was established that pasteurization of milk in cattle leukemia is an integral stage in the overall complex of veterinary and sanitary measures. For pasteurization in livestock farms and milk processing plants, it is necessary to install modern, energy-saving, highly efficient pasteurizers using infrared heating. When using infrared equipment for pasteurization-disinfection of milk ('UOM'), the disinfection of milk occurs at 79.5°C in a stream (without exposure). This mode of milk processing completely destroys the leukemia virus in it and does not affect its nutritional qualities

Keywords: leukemia, milk, pasteurization, temperature, energy consumption, infrared radiation, pasteurizer

Introduction. Today, there is a growing demand for dairy products in the world, which is partly due to increased consumer welfare in developing countries. Global dairy trade accounts for only 6.2% of global production, and demand currently exceeds supply (More, 2009).

The quantity and quality of produced milk is closely dependent on a number of factors, both of endogenous and of exogenous origin (Picinin et al., 2019).

The most important factor that determines the quality, as well as the safety of milk for the consumer, is its receipt from healthy animals (Leitner et al., 2013). During pathological processes in the body of a lactating animal, a number of pathogenic microorganisms are released with milk, that can cause serious harm to the consumer (Chaffer et al., 1999). It should also be noted that these microorganisms often have high antibiotic resistance (Hadzevych et al., 2019).

Monitoring of the total number of microorganisms in relation to the quality of raw milk is mandatory, but determining the presence levels of certain types of bacteria is becoming increasingly important (Nightingale et al., 2008). For example, spores of some spore-forming bacteria present in raw milk at very low levels (< 1/ml) can survive after pasteurization and multiply in dairy and cheese products to levels that lead to their defects (Murphy et al., 2016).

One of the most common and dangerous diseases of oncogenic origin among cattle is leukemia (Frie et al., 2017; LaDronka et al., 2018). Bovine leukemia virus is leukemogenic in at least two mammalian species, it is widespread in commercial dairy herds, and can infect a wide range of *in vivo* hosts and cells, including human cells, *in vitro* (Ferrer, Kenyon and Gupta, 1981). Bovine leukemia virus (BLV), an oncogenic member of the genus *Deltaretrovirus*, is closely related to human T-cell leukemia virus (HTLV-I and II) (Polat, Takeshima and Aida, 2017). There is information that the leukemia virus should be considered as a potential predisposing factor for breast cancer in women (Schwingel et al., 2019).

For the control and prevention of leukemia in different countries, different schemes, technological maps and various veterinary measures that have different efficiencies have been developed (Bartlett et al., 2014; Ruggiero and Bartlett, 2019).

An effective technological stage in the control of animal leukemia and prevention the possibility of its further spread, is milk pasteurization (Baumgartener, Olson and Onuma, 1976). This method is widely used not only in veterinary medicine, but also in humane medicine (Gayà and Calvo, 2018), in the processing industry (Stabel, 2003).

The experiments proved that after pasteurization of milk the number of bacteria is significantly reduced, and pasteurized milk contains an acceptable number of bacteria in more than 90% of the samples. These results indicate that pasteurization may be very effective in reducing bacterial contamination of milk (Elizondo-

Salazar, Jones and Heinrichs, 2010). However, the use of malfunctioning pasteurizers as well as a violation of their operation may lead to risks associated with incomplete disinfection of the processed milk (Fernandes et al., 2015).

World practice shows that it is necessary to observe strictly all existing technological steps in the production of milk (Dong, Hennessy and Jensen, 2012; Paliy and Paliy, 2019).

But above all, in order to receive high quality and safe final product, high-quality raw materials and high-quality equipment are needed, that will not reduce all the properties of whole milk during its processing.

Material and methods. A study on the issue of quality of milk raw materials and equipment used in the pasteurization of milk was carried out. The resistance of pasteurized milk was compared when processing by various methods (storage in a refrigerator at a temperature of 4–5°C). The comparative characteristics and specific energy consumption of the most popular pasteurizer models with 'UOM' milk pasteurizer-disinfectant were described. We studied the specific energy consumption of the 'UOM' units.

Results and discussions. ***Quality raw materials.*** The main suppliers of whole milk to milk processing enterprises are agricultural livestock breeding complexes for cattle. Getting high-quality milk is a laborious and multifactorial process. It should be noted that the faster the milk is cleaned, cooled, and if necessary pasteurized, the more high-quality product will be presented to the milk processing enterprise, and the more profit the farm will receive.

Indicators of milk acidity and bacterial contamination are the most important in determining the grade of milk. To ensure these indicators at the proper level is especially difficult in the warm season. Typically, milk on a dairy farm after receipt is simply cooled and sent to the milk processing plant once a day.

Filtering milk through a non-woven or lavsan filter does not reduce its bacterial contamination, but only reduces the amount of mechanical impurities. It is impossible to cool milk instantly, and the milking process itself lasts 2–3 hours.

All this time, bacterial seeding and the acidity of milk are growing. However, even in pasteurized milk cooled to 4°C, the process of microflora reproduction is slowly continuing. The safety of milk at 4–5°C after infrared (IR) pasteurization is better than after pasteurization in traditional plants (Table 1).

Table 1 — Comparison of the resistance of pasteurized milk after various methods of its processing (storage in the refrigerator at a temperature of 4–5°C)

Pasteurization method	Duration of milk preservation without changing its characteristics, days					
	acidity	taste and smell	resistance	bacterial contamination	by grade	
					class with considering bacterial contamination	acidity
Infrared	15	16	16	3	9	17
Traditional	8	12	14	3	8	11

As a result, it is problematic to deliver milk of highest grade to the dairy plant in the southern regions. In addition, the milk processing enterprise incurs additional costs for heating of pre-refrigerated milk during pasteurization. Such a sales scheme is not optimal for either the farm or the dairy plant.

Using an IR milk pasteurizer-disinfectant ('UOM'), the farm can make more profit due to the higher grade of milk, and the dairy plant, providing itself with high-quality raw materials, heats the milk that has been pasteurized at the supplier's farm not to the pasteurization temperature, but to the necessary for the technological process temperature. The 'UOM' is essential equipment for the prevention of tuberculosis and leukemia during calf feeding. The disinfection mode (80°C without exposure) allows the milk processing enterprise to use milk after UOM for the production of almost all products, because IR pasteurization-disinfection does not change the technological properties of milk, while traditional disinfection (90°C for 5 min) makes it impossible to use milk for the production of a number of products. Since the supply of high-quality raw materials to the dairy enterprise is beneficial both for the farm and for the dairy plant, it became popular to purchase the infrared pasteurizers by dairy plants for the farms-suppliers, which pay off subsequently the plant on the terms agreed upon in the contract.

Quality equipment. Pasteurization as part of the primary processing of milk can be carried out both on the farm and in the milk processing enterprise. In terms of energy consumption, it is more economical to pasteurize milk at the farm before cooling. Table 2 compares the specific energy consumption of the most popular pasteurizer models with 'UOM'.

As can be seen from Table 2, in terms of energy saving, 'UOM' is far ahead of the same well-known designs of pasteurizers. It should be noted that in the table, the energy consumption of traditional pasteurizers with recovery is given for the 'pasteurization' mode, and for 'UOM' — for the 'disinfection' mode.

Table 2 — Comparative characteristics of the specific energy consumption of infrared and traditional pasteurizers, kW×h/t

Type of equipment	Type of technological milk processing operations	Real		Perspective	
		Initial temperature of milk			
		10°C	40°C	10°C	30°C
Traditional capacitive units	without rinsing and cooling	176	132	125	93
	without rinsing with cooling	352	264	250	186
	with rinsing and without cooling	234	176	166	124
	with rinsing and cooling	586	440	416	310
	average value	337	253	239.25	178.25
In-line conventional heat recovery units	without rinsing	38	30	19	15
	with rinsing	51	40	25	20
	average value	44.5	35	22	17.5
'UOM' infrared pasteurizer		Basic design		Promising opportunity	
	without rinsing	15	12	11	8
	with rinsing	19	16	14	10
	average value	17	14	12.5	9

If for traditional pasteurizers a decontamination regime of 90°C with an exposure of 5 min is ensured, their lag behind the 'UOM' will become even more obvious. There are other, little-known models. The main criterion characterizing the price-quality ratio is the number of equipment in operation. Infrared disinfecting pasteurizers have proved themselves as popular, simple, available and reliable equipment.

Infrared equipment for pasteurization-disinfection of milk ('UOM') is currently the only equipment when using which the disinfection of milk occurs at 79.5°C in a stream (without exposure). At a lower pasteurization temperature, 'UOM' works like a regular pasteurizer.

Thus, if 'UOM' is acquired by an economy trouble for tuberculosis or leukemia, it does not have problems with milk intake at the dairy plant, as sparing pasteurization-disinfection mode (79.5°C without exposure) will preserve all technological properties of the product. Both the temperature effect on milk and the mechanical effect are minimized.

If a high-performance 'UOM' is purchased by a dairy plant, milk after pasteurization-disinfection can be packaged (according to the organoleptic characteristics of the product and the safety of the vitamin composition, 'UOM' is superior to traditional pasteurization equipment, but the product has a longer shelf life).

It is also possible to make almost the entire range of dairy products, butter, cream, hard cheeses, casein, milk powder, and baby food from milk that has undergone IR processing. Lines for the production of all of the above listed products are equipped with 'UOM'.

Modern pasteurization equipment may differ in the way the product is heated. Their common (if this is not a bath for prolonged pasteurization) is the presence of a lamellar or tubular recuperator. It is used to reduce the energy consumption and product outlet temperature. If the customer is in danger of getting into the pasteurization equipment milk with high acidity (> 21°T) or milk with a high colostrum content, the following formula is used:

$$t_r = t_i + n_{er} \times (t_p - t_i),$$

where, t_r — product temperature between the recuperator and the heating element;

t_i — initial temperature of the product;

n_{er} — heat exchanger recuperation coefficient;

t_p — pasteurization temperature.

If t_r, as a result of data fitting, is higher than 68°C, there is a risk of coagulation of the product with a high colostrum content or acidity > 21°T in the recuperator. The probability of this is the higher, the closer t_r is to 82°C. As experience shows, sediments are formed on plates in a lamellar and on tubes in a tubular recuperators when milk with a high colostrum content or acidity > 21°T passes even with fluid motion regimes $Re = 9.0 \times 10^3$, where Re is the dimensionless Reynolds criterion. The 'UOM' is equipped with a lamellar (tubular) heat exchanger, the recuperation coefficient of which is 82–89%. Thus, the specific energy consumption per ton of milk with n_{er} = 83%, t_p = 80°C, is 12.5 kW×h/t, with n_{er} = 89% — 9 kW×h/t.

Table 3 (basic design of the 'UOM') indicates the specific energy consumption of the 'UOM'.

Electricity consumption can be reduced by increasing the recuperation coefficient. Recently, the 'UOM' acquisition model has become popular, in which the department of raw materials of a milk processing enterprise, providing itself with quality products, enters into agreements with supplying farms and acquires equipment for them under a leasing scheme. The IR pasteurizer-disinfector is equipped with automation and temperature control (Disk-250 recorder), which ensures reliability of temperature control and the ability to control the supplier by the dairy enterprise.

Table 3 — 'UOM' specific energy consumption

Characteristics	'UOM'-5.0	'UOM'-3.0	'UOM'-2.5	'UOM'-2.0	'UOM'-1.5	'UOM'-1.0
Productivity, l/h	5,000	3,000	2,500	2,000	1,500	1,000
Initial temperature of milk, °C	6–30					
Milk processing temperature, °C at tuberculosis at leukemia	 79 ± 0.5 79 ± 0.5					
During pasteurization	70–80					
Output product temperature, °C	16–40					
Installed power, kW	50	38	34	29	22	15
Specific energy consumption for the processing of one ton of milk, kW×h/t	9–12		11–14		12–15	
Weight of the equipment, kg	700	450	400	350	300	250
Occupied area, m^2	4			3		

A peroxidase test at such a low pasteurization-disinfection temperature will not show high-quality pasteurization. Therefore, either milk is accompanied by a pasteurization thermogram, or the quality of pasteurization is checked by a phosphatase test, or the pasteurization temperature is increased to 86°C.

In leukemia, milk from seropositive animals kept in isolation from the seronegative herd is pasteurized in the farm at temperatures not lower than 80°C (only in this mode it is possible to control the quality of pasteurization by reaction to peroxidase), after which it can be used to feed calves or to send to the dairy enterprise. The milk from the cows of the seronegative herd can be sold to processing plants without preliminary pasteurization. In the case where the leukemia seropositive animals are not separated from the total herd, milk from the entire livestock population of the farm is pasteurized in the specified regimes. In some cases, with the written permission of the Chief State Inspector of Veterinary Medicine in regions, cities and districts, temporarily export of raw milk by separate transport to the dairy plant for technological pasteurization and further processing, is allowed if there is a separate line at the dairy processing plant for receiving such milk.

Milk from seropositive animals kept in isolation from the seronegative herd may be skimmed in the farm. At the same time, only the pasteurized cream is taken to the dairy processing plant, the skim milk is boiled and fed to the animals.

Milk from cows with clinical-hematological signs of leukemia should not be used for nutritional purposes and fed to animals. Such milk is neutralized by the addition of 5% formaldehyde or other disinfectant.

Conclusions. Pasteurization of milk in cattle leukemia is an integral stage in the overall complex of veterinary and sanitary measures. For pasteurization in livestock farms and milk processing plants, it is necessary to install modern, energy-saving, highly efficient pasteurizers for infrared heating. Infrared equipment for pasteurization-disinfection of milk ('UOM') is equipment when using which the disinfection of milk occurs at 79.5°C in a stream (without exposure). This mode of milk processing completely destroys the leukemia virus in it and does not affect its nutritional qualities.

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PORCINE CIRCOVIRUS TYPE II SCREENING IN FERAL SWINE POPULATION IN UKRAINE

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Summary. Porcine circovirus type 2 (PCV2) is an emergent single-stranded DNA virus found all over the world in domestic pigs and wild boars that causes infectious disease with a great impact on swine productivity. PCV2 has 1.7 kb genome that includes two main genes, which encode replication-related protein (*rep*) and the major structural capsid (*cap*) protein. Both of these genes can be used as target sequences for the primer design for the detection of PCV2 as well as for sequencing of designated regions. We carried out a screening due to the PCV2 circulating among the wild boar population in 10 regions of Ukraine. PCR screening was performed using primer pairs designed on the target sequences of the replicative and capsid genes. According to the results of the research, the presence of genetic material of PCV2 was found in 31.8% of the tested samples. The developed set of primers may be suitable for diagnostics, as well as for the development of specific sites for the purpose of sequencing of PCV2 *cap*-gene due to the obtained DNA samples during epizootic screening

Keywords: PCV2, *rep*-gene, *cap*-gene, epizootic screening, PCR

Introduction. Porcine circovirus type 2 (PCV2) is an emergent single-stranded DNA virus found all over the world in domestic pigs and wild boars that causes infectious disease with a great impact on swine productivity (Segalés et al., 2004). Existing studies showed high prevalence of PCV2 infection in pig farms with severe economic losses worldwide.

PCV2 has 1.7 kb genome that includes two main genes, which encode replication-related protein (*rep*) and the major structural capsid protein (*cap*). Both of these genes can be used as target sequences for the primer design for the detection of PCV2 as well as for sequencing of designated regions (Olvera, Cortey and Segalés, 2007).

Previously using *rep*-gene sequences it has been proposed a set of primers for PCR (Gerilovich et al., 2015). It was used to detect 421 bp amplicon with the further conducting of sequencing and phylogenetic analysis. However, according to other investigations, nowadays the most informative and reliable for sequencing is *cap*-gene due to changes in the sequence of the cap gene which are able to cause variability of the capsid protein and pathogenic properties of PCV2 (Guo et al., 2010; Huang et al., 2013). The *cap*-gene is a more reliable phylogenetic marker for PCV2, since it can reconstruct the similar phylogenetic tree as the complete viral genome (Olvera, Cortey and Segalés, 2007).

Therefore, the **purpose of our studies** was to develop a set of primers based on the capsid gene and to compare its effectiveness in detecting PCV2 during surveillance of samples from feral pigs.

Materials and methods. Archival samples of biological material obtained from wild pigs (organs and blood serum), which were received from hunters during the November–December 2012 hunting season, were used for the research.

DNA extraction from biological material was performed using a commercial 'QIAamp cador Pathogen Mini Kit' ('Qiagen', Germany) according to the manufacturer's manual.

PCR was implemented using 'Maxima Hot Start Green PCR Master Mix' ('Thermo Scientific', USA), primers PCV-2 F/R based on *rep*-gene (PVC-2F CGAAGACGAGCGCAAGAAAATACG, PCV-2R CCAATCACGCTTCTGCATTTTCCC) and primers, obtained in this study.

The PCR products were electrophoresed on 1.5% agarose gel ('Sigma') in TAE buffer (0.04 M Tris-acetate, 1 mM EDTA, pH 8) using horizontal electrophoresis chamber ('Bio-Rad', USA).

PCV2 *cap*-gene sequences presented at the NCBI nucleotide database (https://www.ncbi.nlm.nih.gov) were used to select specific primers. All sequences were downloaded, linearized at the same point and aligned into database (Benson et al., 2012).

Multiple sequence alignment and target gene selection were performed using Bioedit 7.0.0 and ClustalW module MEGA 6 (Tamura et al., 2013). The design of primers and their subsequent evaluation by PCR quality criteria, intraspecific specificity and range of detection were performed using AmplifiX 1.5.4 and BLAST-online.

Results. In total 107 samples of biological material were obtained from wild boars during 2012 in 10 regions of Ukraine (Poltava, Sumy, Zaporizhzhia, Chernihiv, Chernivtsi, Cherkasy, Kherson, Lviv, Volyn and Luhansk).

The samples were investigated by the classical PCR using a set of primers used the replicative gene as the target sequence. This primer system flanked a 421 bp variable region of the tail *rep*-gene.

According to the PCR results, 34 (31.8%) samples were determined as positive.

The results of the study of samples of biological material from wild boars for the presence of genetic material PCV2 during the hunting season in 2012 are presented in Table 1.

Table 1 — Results of studies of the biological material samples from feral pigs due to the presence of PCV2 DNA

No.	Region	Number of samples	Positive samples	
			Number	%
1	Poltava	15	3	20.0
2	Sumy	8	5	62.5
3	Zaporizhzhia	13	12	92.3
4	Chernihiv	17	5	29.4
5	Chernivtsi	5	2	40.0
6	Cherkasy	10	1	10.0
7	Kherson	1	0	0.0
8	Lviv	13	3	23.0
9	Volyn	13	1	7.7
10	Luhansk	12	2	16.7
Total		107	34	31.8

The largest number of samples was examined from wild boars from Chernihiv region — 17 samples, Poltava — 15 samples, Zaporizhzhia, Lviv, Volyn — 13 samples each. The lowest number (1 sample) was investigated in the Kherson region. Chernivtsi, Sumy, Cherkasy and Luhansk regions — 5, 8, 10, and 12 samples respectively.

Positive specimens for the presence of PCV2 genetic material were detected in all areas except the Kherson region, that was caused, probably, not by the absence of virus circulation, but by the limited number of samples submitted for study (1 sample).

The largest number of positive samples was found in Zaporizhzhia region — 12 positive out of 13 studied, which was 92.3% and in Sumy — 5 positive out of 8 (62.5%). In other regions of Ukraine this indicator was: Chernivtsi region — 2 samples (40.0%), Chernihiv region — 5 (29.4%), Lviv region — 3 (23.0%), Poltava region — 3 (20.0%), Luhansk region — 2 (16.7%), Cherkasy — 1 (10.0%) and Volyn — 1 (7.7%). In general, this indicator averaged 31.8%, which is similar to the data of other researchers both from Ukraine and from other countries.

Thus, we established the presence of genetic material of PCV2 in samples of biological material from wild pigs at the level of 31.8%.

Positive PCV2 DNA samples obtained from screening studies were used to optimize the application protocol for the further primer selection based on *cap*-gene.

The primers were developed based on the target sequence of the PCV2 capsid gene, which resulted in 95 nucleotide sequences of PCV2 downloaded from on-line databases GenBank. In order to identify conserved sites, optimal search parameters were selected and multiple sequencing of the obtained sequences was performed.

Using BioEdit software option conserved regions were analyzed for the presence of primer sequence candidates. The characteristics of the primers were evaluated by quality parameters: length (base pairs), similarity of melting temperatures (stability), the GC/AT ratio at the 3'-ends, avoiding of duplex formation (Rychlik, 1995).

Based on the results of the studies, a primer pair was selected flanking the 798 bp *cap*-gene region (Table 2).

Table 2 — Primer pair for the 798 bp *cap*-gene region amplification

Primer	Sequence (5'→3')
PCV-2 seqF	CCCATGCCCTGAATTTCCA
PCV-2 seqR1	GCGCACTTCTTTCGTTTTC

Bioinformatic analysis of the developed primers showed their compliance with the requirements for their quality and 100% complementarity with the all PCV2 DNA samples.

For validation of the developed set of primers for their ability to identify the PCV2 DNA at the level of diagnostic, 10 samples of nucleic acids, which during the previous screening tests were positive, and 10 negative samples were selected. 798 bp PCV2 DNA amplicons were obtained by PCR using the developed primers according to the following optimized protocol: denaturation primal at 95°C for 5 min; 40 cycles of template denaturation at 95°C for 30 s; 30 s of primer annealing at 55°C; and 45 s of primer extension at 72°C with a final extension cycle at 72°C for 5 min.

The development of amplicons of appropriate length was confirmed by agarose gel electrophoretic analysis.

According to the results of the studies, it was found that the samples that were positive for the detection of genetic material (samples 1–10) by the primer system for the indication of the PCV2 genome by the *rap*-gene showed the same result when developed primer system was used (Table 3).

Table 3 — Comparative results of application by using primer pairs developed on the different targeted gene sequence (*rep* and *cap*)

Result of studies on the presence of an amplicon of appropriate length on the electrophoregrams										
Number of sample	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
rep-gene (421 bp)	+	+	+	+	+	+	+	+	+	+
cap-gene (798 bp)	+	+	+	+	+	+	+	+	+	+
Number of sample	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**
rep-gene (421 bp)	–	–	–	–	–	–	–	–	–	–
cap-gene (798 bp)	–	–	–	–	–	–	–	–	–	–

This indicates that the set of primers developed by us may be suitable for detecting the genetic material of PCV2 in clinical specimens as well as for amplicon synthetize for sequencing purposes.

Conclusions. Screening studies of samples of biological material from wild pigs from 10 regions of Ukraine were conducted.

According to the results of PCR, 107 samples were examined and the presence of genetic material of PCV2 in 34 samples was established, which was 31.8%. PCR screening was performed using primer pairs designed on the base of the target sequences of the replicative and capsid genes.

According to the results of our research, we have demonstrated the effectiveness of our system of primers to the capsid protein gene in terms of its ability to detect the genetic material of PCV2 in the samples.

Prospects for further research. The developed system of primers may be suitable for diagnostics, as well as for the development of specific sites for the purpose of sequencing of PCV2 *cap*-gene due to the obtained DNA samples during epizootic screening.

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FELINE LEUKEMIAS: FEATURES OF PATHOGENIC CHANGES IN BLOOD

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Summary. Feline viral leukemia is a widespread disease in various countries around the world. According to the data provided by nation-wide data sets, from 3.1% of cats (in the USA) to 24.5% (in Thailand) tested positive for FeLV. In Ukraine, there are practically no results of studying this phenomenon. Leukemia is a malignant blood disease. According to various sources, the animals die within 3–4 years, there is also the possibility of recovery, but the greatest danger is the hidden, chronic course, the virus carriers and the unlimited possibility of spreading the virus, both among domestic and among street animals. The latent course and long incubation period of this disease impede timely diagnosis and effective therapeutic measures. Infection of healthy animals can occur as a result of contact with infected animals, during participation in exhibitions, during the breeding of animals, contacts with stray cats. Manifestations of viral leukemia can vary, affecting primarily the work of the immune system. The pathogenic mechanisms of the disease development should be studied in connection with viral damage to bone marrow stem cells and impaired blood formation processes, the development of an immunodeficiency state. The identification of qualitative and quantitative changes in blood cells and the determination of pathogenic mechanisms of disease development are necessary for early diagnosis of the disease and prevention of infection in healthy animals. Detection of specific signs typical for the leukemic blood picture in cats is a necessary part of a comprehensive diagnosis, together with specific studies (PCR, etc.). A blood test is the primary stage of detecting a disease. Specific structural and functional changes, in particular neutrophils, lymphocytes and erythrocytes, allow us to broaden our understanding of the development of symptoms, the course of the disease and possible outcomes

Keywords: cat, blood smears, erythrocytes, feline viral leukemia virus, immunodeficiency, lymphocytes, neutrophils, pathogenesis

Feline leukemia virus (FeLV) remains one of the most important infectious diseases of cats globally. It manifests primarily through profound anemia, malignancies, and immunosuppression and infects domestic cats and other species of Felidae (Cotter, 2019). Feline retroviruses are widespread infections of cats (Zolototrubov and Fedosov, 2008; Sulimov, 2004).

Leukemia is a malignancy (cancer) of blood cells. In leukemia, abnormal blood cells are produced in the bone marrow. Usually, leukemia involves the production of abnormal white blood cells — the cells responsible for fighting infection. However, the abnormal cells in leukemia do not function in the same way as normal white blood cells. The leukemia cells continue to grow and divide, eventually crowding out the normal blood cells. The end result is that it becomes difficult for the body to fight infections, control bleeding, and transport oxygen.

FeLV is a retrovirus in the subfamily Oncovirinae. Other oncoviruses include feline sarcoma virus, mouse leukemia viruses, and two human T-lymphotropic viruses. Although oncogenesis is one of their more dramatic effects, oncoviruses cause many other conditions, including degenerative, proliferative, and immunologic disorders. Studies show that the prevalence of viral leukemia is up to 30% among urban cats. After the onset of symptoms of a chronic disease, the average life expectancy of animals does not exceed 3–4 years. In the USA, 3.1% of cats in a large, nation-wide data set tested positive for FeLV in 2010, with increased risk among outdoor cats, unneutered males, and cats with other diseases (particularly respiratory disease, oral disease, and abscession). Seroprevalence surveys of varying statistical power have found rates of positive test results to range from 3.6% in Germany and Canada to 4.6% in Egypt and 24.5% in Thailand. Commercial serological sets were used for the examination of 727 cats kept in large towns of the Czech Republic. FeLV antigen and antibodies to FIV were demonstrated in 96 (13.2%) and 42 (5.8%) of the animals, respectively (Knotek et al., 1999). Antigens of FeLV were detected in 16.7% of the sampled cats; 11 (64.7%) of the 17 positive cats were older than one year at the time of testing (Blanco et al., 2009). The prevalence of FIV and FeLV infections in cats in urban settings in Istanbul has been studied (Yilmaz, Ilgaz and Harbour, 2000). The infection of cats with FeLV in the Moscow metropolis was 15.8 % (Gulyukina, 2018). Leukemia of cats is a big problem for nurseries. In nature, FeLV infects domestic cats and a few other Felidae. In the laboratory, cells from a much wider range of species can be infected by some strains of the virus (Turzhanskaya, 2006). For the first time, clinical signs and pathological changes in the body of the Amur tiger, withdrawn from the wild nature, were described (Lyubchenko and Korotkova, 2015).

Leukemia and immunodeficiency as latent virus diseases of cats. To prevent the propagation of these diseases it is recommended to test cats for FeLV and FIV before breeding, hemotransfusion, expositions and moving to another region (Bazhibina and Sokolova, 2010).

The authors of the article did not find data on the distribution of feline leukemia in Ukraine, but according to own research data from the staff of the Department of Pathological Anatomy and Autopsy at the Kharkov State Zooveterinary Academy, only in 2019 this diagnosis was made in several cases. Photos from the research results are shown in Fig. 1–6.

Pathogenesis: the incubation period can last 4–30 weeks. FeLV-related disorders are numerous and include anemia, neoplasia, immunosuppression, immune-mediated diseases, reproductive problems, enteritis, neurologic dysfunction, and stomatitis. The disease has many different clinical symptoms but none of them are specific. Observation of unsuccessful multicat householders has shown that: some of FeLV-positive cats died, other survived and felt not so bad, some of them had different signs of chronic disease (Nepoklonova et al., 2005). There is a great need for sensitive specific methods of screening and identifying carriers and infected with leukemia cats (FeLV) to control the spread of the virus in the cat population. It is known that the cat leukemia virus has tropism for lymphocytes, enterocytes, macrophages, astrocytes, endotheliocytes, etc. (Polyakova et al., 2017).

But what changes are primary? After oronasal inoculation, the virus first replicates in oropharyngeal lymphoid tissue. From there, virus is carried in blood mononuclear cells to spleen, lymph nodes, epithelial cells of the intestine and bladder, salivary glands, and bone marrow. Virus also appears in secretions and excretions of these tissues and in peripheral blood leukocytes and platelets. Viremia is usually evident 2–4 weeks after infection. Six sequential phases of FeLV infection (i. e. viral replication) were identified:

1) lymphoreticular cells in local lymphoid tissues;

2) circulating lymphocytes and monocytes (early cell-associated viremia);

3) lymphoid germinal cells in lymphoid tissues throughout the body;

4) bone marrow neutrophil and platelet precursor cells and intestinal crypt epithelium;

5) circulating neutrophils and platelets (with establishment of viremia);

6) mucosal and glandular epithelial tissues, with excretion of FeLV (Rojko et al., 1979).

Oncogenesis occurs when FeLV virus inserts into the host cellular genome, either in proximity to an oncogene resulting in activation or directly into the oncogene itself to form a recombinant subgroup virus such as FeLV-B that can induce new neoplastic activity in any cell the recombinant virus enters.

In the blood and saliva, the virus is detected one month after infection. After a few months, the pathogen can be found in the bone marrow, spleen and lymph nodes.

In the pathogenesis of the disease one of two ways can prevail: 1) the development of lymphomas and lymphosarcomas in the different organs; 2) direct malignant lesion of the blood-forming apparatus and, as a result, leukemia.

Lymphoma is the most frequently diagnosed malignancy of cats (Levy and Burling, 2019). In virus-positive cats, the complement concentration in the blood decreases, which leads to immunodeficiency and the development of neoplasias (Turzhanskaya, 2006).

Tumors such as lymphoma and lymphoid leukemia develop in as many as 30% of cats with progressive FeLV infections. Regressive infections are also implicated in the occurrence of these tumors in the absence of viremia, but cats with progressive infections may face a 60-fold increased risk of lymphoma development. Most cats with mediastinal, multicentric, or spinal forms of lymphoma are FeLV-positive. However, these forms of lymphoma are becoming less common as the prevalence of FeLV decreases. Karysheva (2002) informs, that in cats, leukemia occurs more often at the age of 1–2 years, and then at the age of 11 years and older, and in almost 90% of the cases animals fall ill on lymphosarcoma. Fibrosarcomas and quasi-neoplastic disorders such as multiple cartilaginous exostoses (osteochondromatosis) may be FeLV-associated.

Primary leukemias are a type of cancer in which abnormal white blood cells displace normal blood cells. This leads to anemia and a lack of normal white blood cells and platelets. Primary leukemias are uncommon, but they have been reported in cats. Retroviruses (feline leukemia virus and feline immunodeficiency virus) are a cause in some cats. Leukemias are classified as acute (sudden and often severe) or chronic (longterm). Acute leukemias, in which the marrow is filled with immature blood cells, generally respond poorly to chemotherapy. In animals that do respond, remission times are usually short. Chronic leukemias, in which there is greatly increased production of one blood cell line, are less likely to cause anemia and are more responsive to treatment.

Diseases associated with FeLV may affect any organ, and include blood dyscrasias (Little et al., 2012; Coleman et al., 2014).

Blood changes can vary and depend on the stage of the disease and the type of hemopoiesis disorders. So it is reported that, in the changes of blood leukocytosis is observed, significant shift of the leukocyte formula to the left, decrease the number of erythrocytes and a gradual decrease in hematocrit (Galatiuk et al., 2016).

Biometric characteristics of the lymphocytes in cats infected with FeLV and FIV are analyzed in comparison with lymphocytes of healthy cats. AFM-scanning revealed significant differences of morphological and biophysical characteristics of lymphocytes of healthy and infected cats (Krasnikova et al., 2016). In sick animals, leukopenia is observed up to 1.5×10^9/l, lymphocytosis is up to 99%, in peripheral blood smears the number of mitotic cells reached 4% (at a rate of up to 0.4%) (Zolototrubov, Fedosov and Grebenshchikov, 2005).

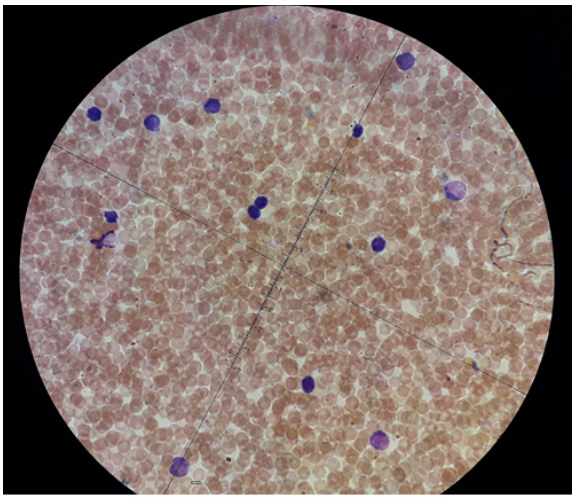

Figure 1. Cat. Blood smear, Pappenheim-Kryukov staining: Acute lymphocytic leukemia

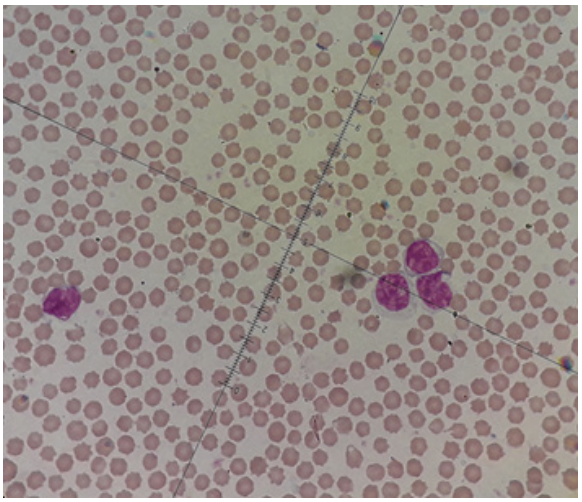

Figure 2. Cat. Blood smear: Acute lymphocytic leukemia, mitotic cells

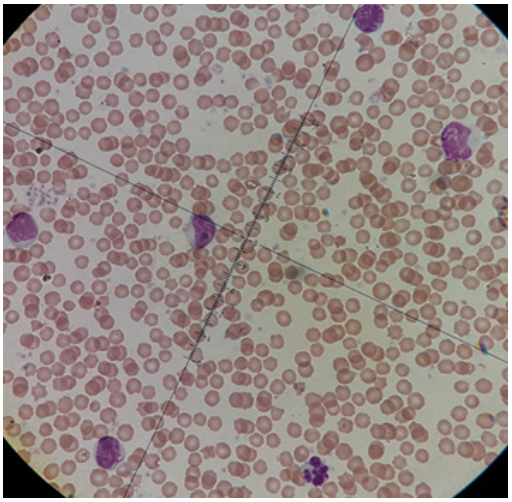

Figure 3. Cat. Blood smear, Romanovsky-Giemsa staining, lymphoblasts

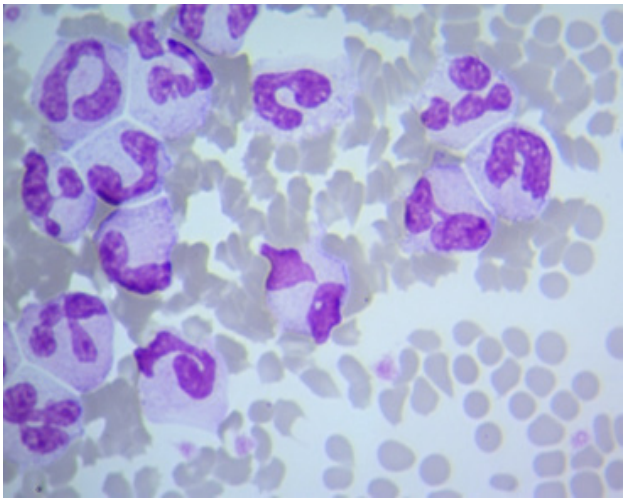

Figure 4. Cat. Giant neutrophils in cat blood in viral leukemia. Disgranulopoiesis

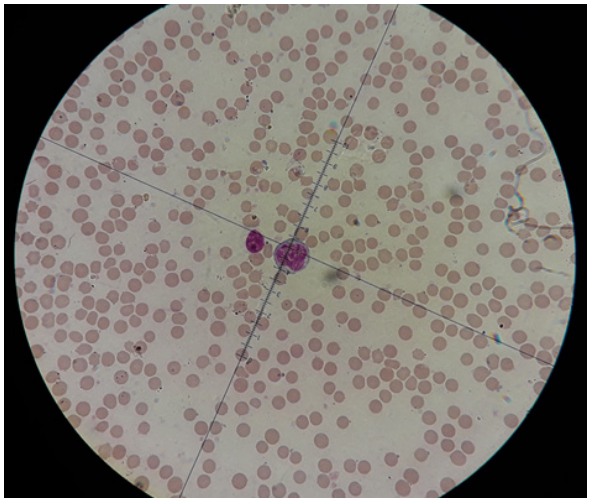

Figure 5. A blood smear of a cat with lymphoma (formation of Heinz bodies in hemolytic anemia)

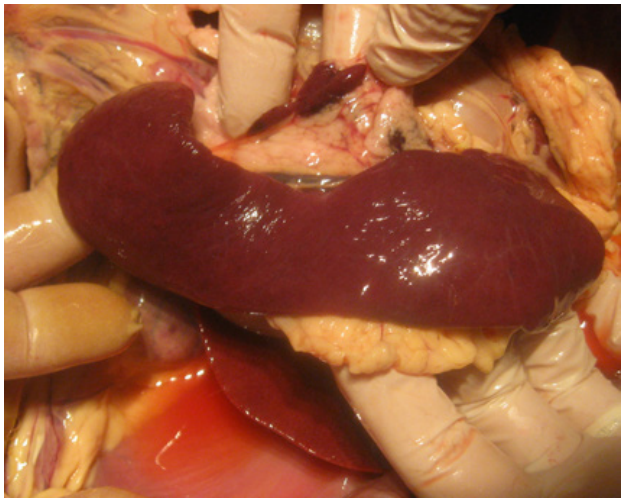

Figure 6. Cat, spleen hyperplasia in leukemia

Figures 1–6. Blood disorders caused by FeLV (all original photo are provided in 2019 by staff of the Department of Pathological Anatomy and Autopsy at the Kharkov State Zooveterinary Academy (Fig. 1–3, 5, 6) and in 2019 (Fig. 4) by Dr. S. V. Shishkanova (2012)

In viral leukemia in cats (Polyakova et al., 2017), lymphocytes can remain within the normal range, as they can either increase pathologically (24% of the entire sample) or decrease (29% of the entire sample).

Leukemia is characterized by the neoplastic proliferation of hematopoietic cells originating in the bone marrow, including neutrophils, basophils, eosinophils, monocytes, lymphocytes, megakaryocytes, and erythrocytes. Feline leukemias are strongly associated with FeLV infection and typically involve neoplastic cells circulating in the blood. Lymphoid leukemias are further classified as acute and chronic. Acute lymphocytic leukemia (Fig. 1–3) is characterized by lymphoblasts circulating in the blood, whereas chronic lymphocytic leukemias have an increased number of circulating lymphocytes with mature morphology.

In animals, there are suppressive disorders of the bone marrow and hematological malignant tumors (Linenberger and Abkowitz, 1995).

Bone marrow failure can lead to nonregenerative anemia and a reduction in the number of all types of blood cells — red, white, and platelets. With widespread marrow involvement, white blood cells are affected first, followed by platelets, and finally red blood cells (Nikitin, 1949; Reagan, Sanders and DeNicola, 2008; Lugovskaya and Pochtar', 2011). Myeloid or myelogenous leukemias are made up of cells that arise from myeloid cells, while lymphoid leukemias arise from lymphoid cells. Knowing the type of cell involved in leukemia is important in choosing the appropriate treatment.

Myelodysplasia (also called myelodysplastic syndrome) is a bone marrow disorder in which growth and maturation of blood-forming cells in the bone marrow is defective. This leads to nonregenerative anemia or shortages of white blood cells or platelets. It is considered a preleukemic syndrome (occurring before leukemia fully develops). Myelodysplasia commonly occurs in cats with feline leukemia but can also occur as the result of other tumors, drug therapy, or mutations in stem cells. Survival rates vary because myelodysplasia can progress to leukemia. Many animals with this state die from infection, bleeding, or anemia.

Myelophthisis is a direct effect of tumors on the bone marrow — replacement of bone marrow spaces with cells that suppress the proliferation of normal hematopoietic cells. When myelophthisis is characteristic of leukemia, disseminated lymphomas (Fig. 2), non-regenerative normocytic normochromic anemia are typical, often in combination with thrombocytopenia and leukopenia.

In the presence of such changes in the clinical analysis of blood, the most important step is the assessment of a venous blood smear for the presence of atypical cells. A cyto- or histological analysis of the bone marrow is also necessary. Fig. 2 shows a bone marrow biopsy from a cat with disseminated lymphoma — a total replacement of bone marrow spaces with large tumor lymphoid cells is noted (hematoxylin-eosin staining, ×400). Anemia occurs in ½–⅓ cats with viral leukemia.

The anemia caused by FeLV is typically nonregenerative and normochromic. Less commonly, macrocytosis or regenerative hemolytic anemia is seen in only 10% of FeLV-induced anemia cases. A blood smear of a cat with leukemia was presented according to the results of own research made by the staff of the Department of Pathological Anatomy and Autopsy. Changes in erythrocytes, anisocytosis and the formation of Heinz bodies during hemolytic anemia are shown (Fig. 5) and hyperplasia of the spleen (Fig. 6). The cause of nonregenerative anemia is usually bone marrow suppression due to viral infection of the hematopoietic stem cells and the supporting stromal cells. Platelet dysfunction, thrombocytopenia, and neutropenia are possible sequelae as well. Tumor-associated anemia can be an important adverse prognostic factor that should be considered when planning therapy targeting the underlying disease (Lisitskaya, 2016). In cats with lymphoma, oxidative red blood cell damage is described with the development of anemia with Heinz's bodies (Fig. 3). A group of researchers reports, at the same time, in animals with pronounced changes in the absolute number of lymphocytes, the hematocrit and the absolute number of red blood cells are often decreased slightly (Polyakova et al., 2017).

Dysgranulopoiesis is characterized by a decrease in the number of progenitor cells in the bone marrow or impaired cell maturation. When conducting a clinical analysis of blood, atypical, immature, giant cells are found in smears (Fig. 4). Giant neutrophils result from missing one of the cell divisions in the bone marrow. These cells can have both a normal morphology of the nucleus, and hyposegmented nuclei. Giant neutrophils always indicate severe neutrophil toxicity. The appearance of giant neutrophils in the blood (Fig. 4) is a characteristic of severe inflammatory processes or disgranulopoiesis, in particular, in viral leukemia in cats (Shishkanova, 2012). The acute stage of FeLV infection occurring 2–6 weeks after infection is rarely detected but typically characterized by mild fever, malaise, lymphadenopathy, and blood cytopenias. Cats unable to mount an adequate immune response become persistently viremic and develop a progressive infection, often leading to fatal disease. Viral suppression of immunity leads to the fact that any infectious disease becomes deadly for an animal. The study of questions of therapy of retrovirus infections is one of major problems of modern medicine (Zolototrubov et al., 2005).

The immunosuppression caused by FeLV creates increased susceptibility to bacterial, fungal, protozoal, and viral infections. Numbers of neutrophils and lymphocytes in the peripheral blood of affected cats may be reduced,

and those cells that are present may be dysfunctional. Many FeLV-positive cats have low blood concentrations of complement; this contributes to FeLV-associated immunodeficiency and oncogenicity, because complement is vital for some forms of antibody-mediated tumor cell lysis.

A deficiency in phagocytosis can be caused by a low number of phagocytes in the blood or by a defect in their ability to act normally. The deficiency increases susceptibility to bacterial infections of the skin, respiratory system, and gastrointestinal tract. Stomatitis (Fig. 5) is more classically associated with FIV infection, but FeLV infection can also predispose cats to chronic ulcerative proliferative gingivostomatitis. Clinical sequelae include pain, anorexia, and tooth loss. An immune-mediated mechanism is likely, particularly in combination with coinfections such as feline calicivirus.

Immune complexes formed in the presence of moderate antigen excess can cause systemic vasculitis, glomerulonephritis, polyarthritis, and a variety of other immune disorders. In FeLV-infected cats formation of immune complexes under conditions, in which FeLV antigens are abundant and anti-FeLV IgG antibodies are sparse, leads to the development of immune-mediated diseases.

Coinfection with FeLV and feline panleukopenia virus (FPV) has been implicated in feline panleukopenia-like syndrome (FPLS), which is also termed FeLV-associated enteritis. FPLS resembles feline panleukopenia both clinically and histopathologically and is characterized clinically by progressive anorexia, depression, vomiting, hemorrhagic diarrhea, weight loss, gingivitis, oral ulceration, severe neutropenia, and septicemia (MacPete, 2014; Powers et al., 2018). FPV antigen is inconsistently present on diagnostic testing in these cases, and the pathogenesis and exact role of each virus in the development of this syndrome are incompletely understood.

Conclusions. Pathogenetic features of blood changes in viral leukemia of cats manifest as a result of damage to the cells of the hematopoietic apparatus. When the myeloid form in the peripheral blood among atypical cells, giant immature neutrophils are predominantly detected. Lymphoid form can be manifested by lymphocytosis.

The result of the primary violation of blood formation is anemia and immunodeficiency, which is manifested by a decrease in antitumor protection, a violation of the barrier mechanisms of mucous membranes and the skin and, as a consequence, secondary infection and the occurrence of tumors in various organs.

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Part 2. Biotechnology and genetics

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DEVELOPMENT OF A MULTIPLEX PCR TEST-SYSTEM FOR DETECTION OF BHV-1, BVDV, *CHLAMYDIA* SPP. AND *MYCOPLASMA* SPP.

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Summary. This study describes development of a multiplex PCR assay for detection of BHV-1, BVDV, *Chlamydia* spp. and *Mycoplasma* spp. infections in bovines. The assay was developed using genomic DNA and RNA and four sets of PCR primers targeting 16S rRNA genes of *Chlamydia* spp., *Mycoplasma* spp., 5'-UTR of Bovine viral diarrhea virus, gE of Bovine herpesvirus-1, respectively. A total of 100 tissue samples were collected from cattle suspected to be infected with the viral and bacterial pathogens (BVDV, BHV-1, *Chlamydia* spp. and *Mycoplasma* spp.) from different regions of Ukraine. A part of sample was stored at –50°C for isolation of genomic DNA and RNA. The multiplex PCR assay was optimized in the study. The specific primers designed and used in the study were found sensitive and specific in amplifying target genes viz. 16S rRNA, gE, 5'-UTR of *Chlamydia* spp., *Mycoplasma* spp., BHV-1 and BVDV, respectively. The PCR primers used in the optimization of multiplex PCR assay for detection of Bovine viral diarrhea virus, Bovine herpesvirus-1, *Chlamydia* spp., *Mycolasma* spp. could amplify 221 bp, 111 bp, 386 bp, 279 bp products, respectively. Non specific amplification was not observed

Keywords: Bovine herpesvirus-1, Bovine viral diarrhoea virus, *Chlamydia*, *Mycoplasma*, multiplex PCR, bovines

Introduction. In veal production, bovine respiratory disease (BRD) is the most common and economically important disease. BRD is a multifactorial disease, involving multiple potentially pathogenic microorganisms that causes economic losses due to morbidity, mortality, medication costs, increased time on feeding and associated labor costs (Wisselink et al., 2017; Griffin, 2014; Snowder et al., 2006).

Bovine respiratory disease appears to be precipitated by an imbalance in the triad of interaction among one or more infectious agents, host defenses, and environmental stressors. Viruses isolated from cattle with BRD include Infectious bovine rhinotracheitis virus ((IBRV), Bovine herpesvirus-1 (BHV-1)), Bovine viral diarrhea virus (BVDV), Bovine respiratory syncytial virus (BRSV), parainfluenza-3 virus (PI-3V), BHV-4 and others. Bacterial pathogens associated with BRD include *Pasteurella haemolytica* Al (now *Mannheimia haemolytica*), *Pasteurella multocida* A:3, *Haemophilus somnus*, *Actinomyces pyogenes*, *Mycoplasma bovis*, *Mycoplasma dispar*, *Mycoplasma hyorhinis*, *Ureaplasma diversum, Chlamydia* spp., etc. (Bowland and Shewen, 2000). Viral and bacterial pathogens together with mycoplasma and environmental risk factors are the most common cause of diseases, ranging from common colds to life-threatening pneumonia. A large number of both RNA and DNA viruses uses the respiratory tract to initiate host infection (Paller et al., 2017).

Infectious abortion is a significant cause of reproductive failure and significant economic losses for the cattle industry. Under optimal laboratory conditions, etiologic diagnosis is achieved in 23.3 to 45.5% of the cases. The variety of infectious agents have also been reported to cause bovine abortion throughout the world, agents such as *Chlamydophila psittaci*, *Mycoplasma bovis*, *Mycoplasma bovigenitalium*, *Ureaplasma diversum*, Bovine viral diarrhoea virus and Bovine herpesvirus-1 and others (Selim, Elhaig and Gaede, 2014; Maunsell et al., 2011; Miles, 2009; Ellis, 2009).

The purpose of this study was to develop multiplex PCR for detection of four pathogens in a single reaction with high sensitivity and specificity using a DNA template extracted directly from tissue samples.

Materials and methods. ***Collection and screening of clinical materials.*** A total of 100 tissue samples were collected from cattle suspected to be infected with viral and bacterial pathogens (BVDV, BHV-1, *Chlamydia* spp. and *Mycoplasma* spp.) in different regions of Ukraine. A part of each sample was stored at –50°C for isolation of genomic DNA and RNA.

Designing of primers for multiplex PCR. The 16S rRNA, 5'-UTR, gE genes sequences available from public databases GenBank (http://www.ncbi.nlm.nih.gov) were analyzed with BioEdit 7.2.0. PCR primers were derived from these regions with AmplifX primer analysis software (Table).

Table — List of primers used in multiplex PCR

No.	Oligomer name and sequences (5'-3')	Amplicon size
1	*Chl*_F GCGTGTAGGCGGAAAGGAAAGTTA *Chl*_R AAACCACATGCTCCACTGCTTG	386 bp
2	*Myco*_F TGTTTAGCGGGGTTGAGAGACTGA *Myco*_R TTCCGGATAACGCTTGCAACCT	279 bp
3	BHV_F ACGCGGCCATTACAAACCAGTACA BHV_R TGCGCAGGTACTCGGCTTT	111 bp
4	BV_F TGGGAGGTGGAACATGGTGTCATA BV_R ACTAGTTCGATGAGCCTGGTCAGA	221 bp

Our primer design was based of the 16S rRNA, 5'-UTR, gE genes sequences with BioEdit software (http://www.mbio.ncsu.edu/Bioedit/bioedit.html) using CLUSTAL W. The specificity of the primer pairs was also assessed against the NCBI nucleotide sequence database (ftp://ftp.ncbi.nih.gov/blast/db/FASTA). All the primers were checked for their properties like Tm value, length, presence of self and cross dimer using oligonucleotide analyzer software and were also checked for their specificity in BLAST tool of NCBI before custom synthesized.

Optimization of the multiplex PCR assay. PCR conditions were optimized as initial denaturation (95°C for 2 min), for 5 min followed by cycles of denaturation (95°C for 1 min), primer annealing (60°C for 1 min) and extension (72°C for 1 min) along with a final extension of 72°C for 4 min. Reaction was performed in automatic thermal cycler ('Bio-Rad', USA) and total 10 µl PCR product of each sample was electrophorosed in 1.5% agarose gel containing 10 µl/ml ethidium bromide. PCR amplification was visualized under UV light.

Results. ***Optimization of multiplex PCR.*** The PCR primers used in the optimization of multiplex PCR assay for detection of Bovine viral diarrhoea virus, Bovine herpesvirus-1, *Chlamydia* spp., and *Mycoplasma* spp. could amplify 221, 111, 386, and 279 bp products, respectively. Non specific amplification was not observed (Fig.).

Validation of mPCR assay using field samples. DNA and RNA samples (40 known positive and 70 negative samples by using simple PCR), subjected to mPCR revealed comparatively high infection rate for single (35%) as well as for mixed infection (5%). Occurrence of single species infection detected by mPCR was 20% for *Mycoplasma* spp. (20/100), 7% for BVDV (7/100), 5% for *Chlamydia* spp. (5/100), and 3% for BHV-1 (3/100). However, mPCR revealed higher number of mixed infections of BHV-1, BVDV (n = 2), BHV-1, BVDV, *Mycoplasma* spp. (n = 3). Further, all samples tested positive by simple PCR, were also found positive in mPCR with hundred percent agreements.

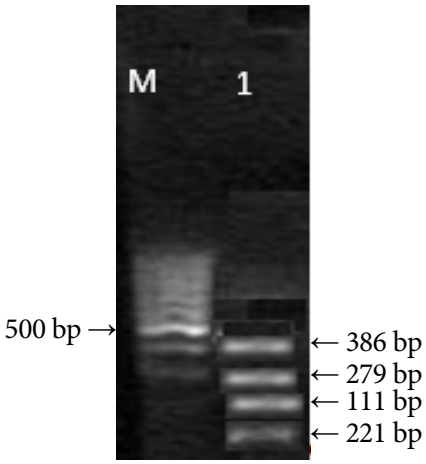

Figure. Standardization of mPCR using positive DNA and RNA of Bovine herpesvirus-1, Bovine viral diarrhoea virus, *Chlamydia* spp., *Mycoplasma* spp. (Lane M: 100 bp DNA ladder, Lane 1: amplicons of 386 bp of *Chlamydia* spp., 279 bp of *Mycoplasma* spp., 221 bp of Bovine viral diarrhoea virus, 111 bp of Bovine herpesvirus-1)

Discussion. In fact, there are many reports available on the development of PCR assays for detection of single pathogens (bacterial or virus) infecting ruminants (Herilovych, Stehnii and Kucheriavenko, 2007; Stehnii, Kovalenko and Kovalenko, 2004).

Some researchers have also developed duplex PCR assays for detection of co-infection of two pathogens infection of bovines (Stehnii et al., 2014).

Detection of multiple pathogens in a single tube will be more convenient for field diagnosis, than the single or duplex PCR assays. Limited reports are available for the development of multiplex PCR. The multiplex PCR assay was optimized in the study.

The specific primers designed and used in the study were found sensitive and specific in amplifying the target genes viz. 16S rRNA, gE, 5'-UTR of *Chlamydia* spp., *Mycoplasma* spp., BHV-1, and BVDV, respectively. Moreover, the size of amplicons ranged between 111 and 386 bp, and the difference between any two fragments ranged between 50 and 100 bp, facilitating easy differentiation of bands on gel electrophoresis.

Conclusion. These results revealed that mPCR developed in the study was capable of specific and sensitive detection of single as well as multiple species infection in cattle.

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Part 3. Biosafety

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DETERMINATION OF TOXICITY INDICATORS AND ASSESSMENT OF THE SENSIBILIZING ACTION OF THE PREPARATION FOR THE EXTERNAL USE 'OINTMENT FOR WOUNDS'

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Summary. The purpose of the work is a determination of toxicity and sensitizing effects of a new external preparation 'Ointment for wounds', based on the essential oils of Siberian pine, eucalypt, tea-tree, cedar, clove, and oil solution of chlorophyllite. The experimental study was performed on rats weighing 160–190 g, 2–3 months of age and mice weighing 18–21 g, 3 months of age. At the stages of preclinical study, it was determined acute and subacute effects of the drug, when administrated on the skin and directly into the stomach of experimental animals. The skin-irritant effect of the phytopreparation was investigated when applied to the skin and buccal mucous membrane. The sensitizing effect of the ointment was evaluated by reproducing local reactions. The acute toxicity assessment was performed on the survival rate of laboratory animals after oral administration of the preparation at doses from 5,000 to 25,000 mg/kg of body weight, with 5,000 units increments. Subacute toxicity in the experiment was evaluated by the dynamics of morphological and biochemical parameters of blood and the study of the coefficients of laboratory animals' internal organs mass. It was found that the introduction of the drug 'Ointment for wounds' in the stomach did not cause significant changes in the behavior of rats, all animals remained alive. Studies have shown the absence of skin-irritant effect of the preparation, as well as the absence of irritant effect on the buccal mucous membrane. Even in a long-term experiment to study subacute toxicity when applied to the skin, no toxic effects of the preparation on the basis of essential oils and oil solution chlorophyllite of were found. The index sensitizing effect of the preparation was less than one, indicating the absence of sensitizing effect. According to the classification of substances by toxicity and danger (requirements of SOU 85.2-37-736:2011 and GOST 12.1.007-76) belong to hazard class IV. In general, 'Ointment for wounds' does not have a toxic effect on the functions of vital organs, and at repeated administration is almost harmless

Keywords: essential oil, chlorophyllite, toxity, rats, mice, skin-irritant effect, sensitizing action, morphological composition of blood, transaminase activity

Introduction. The market of veterinary dermatological medicines in Ukraine is presented mainly by expensive foreign drugs. It became necessary to expend the range of drugs with the latest developments (Bushuieva, 2013; Gerecke, 2005).

Popular multicomponent drugs — derivatives of antibiotics and corticosteroids, do not always act adequately at all levels of the pathological process. Considerable attention of domestic and foreign researchers is focused on the search for highly effective and environmentally safe therapeutic and preventive agents used in skin diseases in animals (Horiuk, 2018; Shaheen, Tantary and Nabi, 2016).

Recent trend is the use of biologically active components of plant origin as an alternative to antibiotic therapy (Tamminen, Emanuelson and Blanco-Penedo, 2018; Vorobets et al., 2018). Successfully selected combinations of essential oils are often no less effective than synthetic biocides, and the risk of the emergence of resistant strains of microorganisms is reduced (Sobrinho Santos et al., 2019; Queiroga et al., 2018).

The cost of veterinary drugs and medical measures with the use of raw materials of natural origin is more attractive. In dermatology, the combination of antimicrobial, anti-inflammatory, immunomodulatory and reparative properties is the main criterion for the selection of biologically active substances of plant origin (Wolski et al., 2017).

Among the herbal preparations, the essential oils of Siberian pine (Carrión-Prieto et al., 2018; Shpatov et al., 2017), eucalyptus (Adnan, 2019; Harkat-Madouri et al., 2015), clove (Packyanathan and Prakasam, 2017), cedar (Bennouna et al., 2018), tea-tree (Li et al., 2016; Smith et al., 2014) and oil solution of chlorophyllite have the above mentioned pharmacological characteristics. The possibility to combine the diverse properties of plants in a single therapeutic agent based on the listed above essential oils and oil solution of chlorophyllite became the basis for the creation of an experimental drug in the form of ointment.

A prerequisite for the registration of new medicines is a preclinical testing in laboratory animals to determine the

nature and severity of the possible harmful effects of the drug on the body, and in particular to determine the toxicity and sensitizing effects (Kosenko et al., 1997; Kotsiumbas et al., 2006).

The purpose of the work is to determine toxicity indicators and to evaluate the sensitizing effect of a new drug for external use 'Ointment for wounds'.

Materials and methods. For experimental evaluation of the toxic-hygienic parameters of the medicinal product 'Ointment for wounds' sexually mature animals were used: rats weighing 160–190 g, aged 2–3 months, and mice, weighing 18–21 g at the age of 3 months, which were maintained in the vivarium of the Research Epizootology Station of the Institute of Veterinary Medicine of the National Academy of Agrarian Sciences of Ukraine (Rivne). The animals were kept on a standard diet according to the requirements of sanitary and hygienic standards, and received food and water *ad libitum*. All experiments were conducted in accordance with the guidelines 'Preclinical Studies of Veterinary Medicines' (Kotsiumbas et al., 2006) and 'Methodological Guidelines for Toxicological Evaluation of Chemicals and Pharmacological Preparations Used in Veterinary Medicine' (Vysotskiy et al., 2007). The animals were housed in a test room, in standard cages, under natural day-night light mode, at temperature 20–25°C, humidity not more than 55%. The basis of the diet was pelleted feed that has been manufactured according to the 'Scientific and Practical Recommendations for Laboratory Animals Keeping and Work with Them' (Kozhemiakin et al., 2002). All manipulations with experimental animals were conducted in accordance with the rules of the 'European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes' (CE, 1986) and Council Directive 86/609/EEC (CEC, 1986). The study was conducted in four stages.

In the first stage, study of ointment acute toxicity was performed on 40 white outbred rats of both sexes, weighing 160–190 g. The drug was administered to the animals once, using an oral probe, at doses from 5,000 to 25,000 mg/kg, with an interval of 5,000 units (by the absolute weight of the preparation). Each dose was examined in 8 animals. The control group (5 animals) was also injected intragastrically with water. Rats did not receive food for the night preceding the test and for 3 hours after administration of the drug. Observations on the animals were carried out for the next 14 days.

In the second stage, subacute toxicity was studied. The study was performed on 20 white rats (2–2.5 months of age, both sexes equally). The drug 'Ointment for wounds' was administered to animals daily intragastrically on an empty stomach, in the form of an aqueous emulsion at a dose of 0.4 ml of the drug per animal. The control group of rats received water intragastrically. The duration of the experiment was 21 days. Then, animals under brief ether anesthesia were euthanized with instantaneous one-stage decapitation, organs (liver, kidney, heart, lungs, spleen, pancreas) were removed, and weighed on torsional scales and body mass coefficients were calculated (Kosenko et al., 1997).

In addition, blood samples were taken to assess subacute toxicity of the drug. The blood was tested by conventional methods for the concentration of hemoglobin, the number of red and white blood cells, the hematocrit volume. The activity of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), as well as the content of total protein, urea and glucose in the serum were determined using the biochemical analyzer 'Cobas c 311' (Roche Diagnostics, Switzerland).

The third stage of the study involved the study of skin irritation: it was determined on 12 white rats by rubbing of the test sample of the ointment, emulsified with water in a ratio of 1:10, in the shaved areas for 30 days. Changes in the functional state of the skin of experimental animals were determined by the degree of inflammatory reaction. Assessment of the skin was performed on the 10^{th}, 20^{th}, and 30^{th} days of the study.

In the fourth stage, sensitizing effect of the ointment according to the local reactions was examined. The study was conducted in 6 white rats, aged 2.5 months. Ointment, emulsified with sterile water in the ratio of 1:10, was introduced once, at a dose of 0.2 ml, in the area of the transitional fold of the oral cavity, on the submucosal layer of the mucous membrane.

The results were evaluated in points given in Table 1.

Table 1 — Evaluation of the sensitizing effect of the preparation in points

0 points	No inflammation (no sensitizing effect) (1s = 0)
1 point	Slightly noticeable inflammation (mild sensitizing action) (1s = 1)
2 points	Well-marked inflammation (moderate sensitizing action) (1s = 2)
3 and 4 points	Brightly marked inflammation (expressed sensitizing action) (1s = 3)

Statistical processing of the results was performed by variation statistics using Statistica 6.0 (StatSoft, USA). Nonparametric research methods were used (Wilcoxon-Mann-Whitney test). The arithmetic mean (x), standard error of the mean (SE) was determined. The difference between the two averages was considered statistically significant when: * — $p < 0.05$, ** — $p < 0.01$, *** — $p < 0.001$.

Results and discussion. According to the results of the first stage it was found that after intragastric administration of the drug in doses of 5,000, 10,000,

15,000, 20,000, and 25,000 mg/kg the signs of intoxication in rats were not observed: animals were clean, active, had a satisfactory appetite, responded to sound and light stimuli. The processes of urination and bowel movements were normal. Respiratory disorders and convulsions were not noted. The condition of hair, skin, mucous membranes remained physiologically normal throughout the observation period. Reflex excitability in all animals was maintained. During the experiment, no lethal outcomes were reported in any group (Table 2).

Table 2 — Results of the study of acute toxicity of 'Ointment for wounds', when administered intragastrically

Group No.	Sex	Dose, mg/kg	Died rats/ surviving rats
1	males	5,000	0/4
	females		0/4
2	males	10,000	0/4
	females		0/4
3	males	15,000	0/4
	females		0/4
4	males	20,000	0/4
	females		0/4
5	males	25,000	0/4
	females		0/4

In the second stage, the composition of the peripheral blood, biochemical parameters were investigated and the coefficients of the mass of the internal organs were determined under the conditions of the subacute action of the preparation for external application 'Ointment for wounds'. Studies of rat blood found that in rodents of the experimental group, hemoglobin and red blood cells significantly exceeded control group, by 13.9 and 7.6%, respectively, whereas hematocrit and white blood cells decreased relative to controls ($p < 0.001$) by 4.6 and 10.4%, respectively (Table 3).

Table 3 — Effect of administration of 'Ointment for wounds' on blood hematological parameters of rats (M ± m, n = 10)

Parameters	Group of animals		Reference value
	Control	Experimental	
Hemoglobin, g/l	129.2 ± 1.49	147.2 ± 1.12***	120.00–150.00
Red blood cells, T/l	6.03 ± 0.05	6.49 ± 0.1**	6.00–7.80
Hematocrit, %	37.2 ± 0.11	35.5 ± 0.17***	35.00–47.00
White blood cells, g/l	6.82 ± 0.03	6.11 ± 0.12***	5.9–13.2

Notes: ** — $p < 0.01$, *** — $p < 0.001$.

It should be noted that fluctuations in peripheral blood composition were within the reference levels (Trakhtenberg et al., 1991; Abrashova et al., 2013), and the obtained data indicate a possible stimulatory effect of the drug on the organs of hematopoiesis, and the absence of toxic effect when the drug was administered intragastrically.

In the study of biochemical parameters of white rats' blood, a significant excess of all investigated parameters relative to the control group was established: total protein — by 16.5%, ALP — by 10.1%, ALT — by 12.2%, and AST — by 12.3% (Table 4), however, these indicators were also within the reference levels (Trakhtenberg et al., 1991; Abrashova et al., 2013), which on the one hand indicates the absence of toxic actions for the introduction of the drug 'Ointment for wounds', and on the other — it stimulates the hepatobiliary system.

Table 4 — Biochemical parameters of white rats' blood on the 21st day of the experiment (M ± m, n = 10)

Parameters	Group of animals		Reference value
	Control	Experimental	
Total protein, g/l	51.6 ± 0.29	60.1 ± 0.15***	50.0–70.0
ALP, U/l	231.0 ± 3.0	254.4 ± 4.2***	220.0–330.00
ALT, U/l	52.6 ± 0.7	59.0 ± 0.51***	50.0–70.0
AST, U/l	119.0 ± 0.62	133.6 ± 2.07***	100.0–140.0

Note: *** — $p < 0.001$.

After calculating the coefficients of the mass of the internal organs, it was found that the coefficient of lung mass was higher than the control by 9.3% ($p < 0,05$), and the kidney — lower by 10.1%, whereas the values of the coefficients of weight of liver, heart and spleen did not differ from those in the animals of the control group (Table 5), which confirms the absence of toxic action of the drug 'Ointment for wounds', and indicates the development of adaptogenic processes in the rats of the experimental group.

Table 5 — Mass ratios of white nonlinear rats' internal organs on day 21st of the study of subacute toxicity of the preparation 'Ointment for wounds' (M ± m, n = 10)

Internal organs	Group of animals	
	Control	Experimental
Lungs	1.08 ± 0.03	1.18 ± 0.02*
Liver	5.30 ± 0.19	5.06 ± 0.1
Kidneys	0.99 ± 0.01	0.89 ± 0.01**
Heart	0.6 ± 0.01	0.56 ± 0.01
Spleen	0.45 ± 0.01	0.48 ± 0.02

Notes: * — $p < 0.05$, ** — $p < 0.01$ relative to control.

According to the results of the third stage of the research, it was found that the index of skin-irritant action of the medicinal product 'Ointment for wounds' was 0 points, i. e. no skin-irritant action was established.

The results of the study of the sensitizing effect of the preparation 'Ointment for wounds' (the fourth stage of research) are shown in Table 6.

Table 6 — Evaluation of the sensitizing effect of 'Ointment for wounds' (M ± m, n = 3)

Animal group	Sensitizing effect index
Control	0.33 ± 0.04
Experimental	0.67 ± 0.05*

Note: * — $p < 0.05$.

In the experiment, the index of sensitizing action of the medicinal product 'Ointment for wounds' was less than one, although it probably two times exceeded the control, which on the one hand indicates the absence of sensitizing effect of this drug, and on the other — stimulating effect on the immune system of rats.

Thus, according to the results of toxicological studies, analysis of possible changes in the structure and functions of vital organs, the study of experimental animals' blood parameters, we can conclude that there is no toxic effect, locally irritating and sensitizing effect of the drug «Ointment for wound», both after oral administration and after dermal application, i. e. it is harmless.

Conclusions. 1. The results of acute toxicity studies indicate that the LD_{50} for the drug 'Ointment for wounds', when administered intrgastrically, is greater than 25,000 mg/kg (the absolute mass of the drug). Intragastric administration of the maximum dose of the preparation did not have any negative effects.

2. Under the conditions of subacute action study of the drug 'Ointment for wounds' after external application in the experimental group of rats, significant changes in hematological, biochemical parameters of blood were found, but they were within the reference levels and weight coefficients of internal organs, indicating the absence of toxic effects of the drug and the development of adaptogenic processes in rats of the experimental group.

3. The index of skin-irritant effect of the medicinal product 'Ointment for wounds' was 0 points, and the index of sensitizing effect of the drug was less than one for the experimental group (although it probably two times exceeded the control), indicating the absence of skin-irritant and sensitizing effect, and the stimulating effect on the immune system of rats.

4. According to the classification of substances by toxicity and danger (requirements of SOU 85.2-37-736:2011 and GOST 12.1.007-76) the preparation 'Ointment for wounds' refers to the IV class of toxicity — low toxic substances and the IV class of danger — low-hazard substances.

5. The obtained results indicate the feasibility of further clinical studies on target animals due to safety of the preparation.

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VIRUCIDAL PROPERTIES OF INNOVATIVE DISINFECTANT TO AVIAN INFLUENZA VIRUS AND NEWCASTLE DISEASE VIRUS

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Summary. The first and the main link in the system of prevention of the occurrence and distribution of avian influenza and Newcastle disease is monitoring and the effective prophylaxis of the above diseases. At the same time the conducting of disinfection of the objects of veterinary control is an important stage in the system of veterinary and sanitary measures. A number of disinfectants that contain different classes of chemical compounds as active substances have been developed and proposed for practical use. The large-scale production of disinfectants and their introduction into practice is impossible without the preliminary laboratory assessment of their antimicrobial properties, the determination of the spectrum of their biocidal effect and physical, chemical and toxicological properties. The aim of our work was to study the virucidal properties of a new aldehyde disinfectant using the test models of the viruses of Newcastle disease and avian influenza. The experiments to study virucidal properties of the disinfectant regarding the viruses of avian influenza and Newcastle disease have been carried out at the Department for Avian Diseases Study of the National Scientific Center 'Institute of Experimental and Clinical Veterinary Medicine' in accordance with the guidelines 'Methods for determining and evaluating the safety and quality of disinfectants and disinfecting detergents used in the production, storage, transportation and sale of products of animal origin' (Kotsiumbas et al., 2010). The determination of the virucidal properties of the disinfectant has been conducted in two stages: stage 1 — the determination of the virucidal activity of the product by the suspension method; stage 2 — determination of the virucidal activity of the product on test objects. As a result of the research conducted by the suspension method the presence of the virucidal properties of the innovative disinfectant (the mixture of quaternary ammonium compounds — 25%, glutar aldehyde — 11%, isopropanol, non-ionic surfactants) for the viruses of avian influenza and Newcastle disease has been determined. It has been found that the above preparation completely inactivated the infective properties of viruses when used in the concentration of 0.1%, with the interval of 30 minutes and in the concentration of 0.5% — 15-minute interval. It has been proved that the use of the disinfectant in 0.1% concentration for 30 minutes disinfected the test objects (wood, metal, tile, textile) that were contaminated by the pathogenic agents of avian influenza and Newcastle disease

Keywords: disinfectant, virus, virucidal properties, test object, chick embryos, concentration, exposition

Introduction. At present infectious diseases of viral etiology are of great danger for industrial poultry farming. The above diseases can affect the susceptible animals and the infection can spread over the large territory very quickly. Newcastle disease (ND) and avian influenza are among the most dangerous and economically important actual diseases (Capua and Marangon, 2000; Camenisch, Bandli and Hoop, 2008; Mehrabanpour et al., 2011).

It has been known that Newcastle disease is not dangerous for human health but it is of great epizootological importance for poultry farming. In spite of the fact that the disease has been known for a long time, and the main peculiarities of its agent and pathogenesis have been studied very well, and the methods of the disease diagnosis and specific prevention have also been developed, the outbreaks of the disease are regularly registered in different countries of the world and they bring great economic losses to the farms (Liu et al., 2008; Hoque et al., 2012).

Avian influenza also causes great economic losses to poultry farming both in the developed countries and in the developing ones. Much attention in the world is paid to influenza viruses of type A because the most dangerous and highly pathogenic viruses belong to this type (Capua et al., 2000; Abolnik et al., 2009).

As a result of the analysis of the present epizootic situation regarding highly pathogenic avian influenza and the risks for poultry farming in Ukraine it has been stated that avian influenza and its highly pathogenic variants pose a real threat for the industrial and private poultry farming with serious economic consequences in case of the pathogen occurrence in the farm (Stegniy, Muzyka and Pishchanskyi, 2018).

The first and the main link in the system of prevention of the occurrence and distribution of avian influenza and Newcastle disease is the monitoring and the effective prophylaxis of the above diseases. At the same time, the disinfection of the objects of veterinary control is an important stage in the system of veterinary and sanitary measures (Paliy et al., 2018; Paliy and Stegniy, 2018).

A number of disinfectants that contain different classes of chemical compounds as active substances have been developed and proposed for practical use, and they are recommended for use in different branches of industry (Zavgorodniy and Paliy, 2014; Paliy et al., 2015; Kovalenko, Paliy and Zagrebelniy, 2017; Rodionova and Paliy, 2017).

It should be pointed out that the large-scale production of disinfectants and their introduction into practice is impossible without the preliminary laboratory assessment of their antimicrobial properties, the determination of the spectrum of their biocidal effect and physical, chemical and toxicological properties (Zavgorodniy et al., 2013; Paliy et al., 2016).

The aim of our work was to study the virucidal properties of a new aldehyde disinfectant on the test models of the viruses of Newcastle disease and avian influenza.

Materials and methods. The experiments to study virucidal properties of the disinfectant regarding the viruses of avian influenza and Newcastle disease have been carried out at the Department for Avian Diseases Study of the National Scientific Center 'Institute of Experimental and Clinical Veterinary Medicine' in accordance with the guidelines 'Methods for determining and evaluating the safety and quality of disinfectants and disinfecting detergents used in the production, storage, transportation and sale of products of animal origin' (Kotsiumbas et al., 2010). The determination of the virucidal properties of the disinfectant has been conducted in two stages: stage 1 — the determination of the virucidal activity of the product by the suspension method; stage 2 — the determination of the virucidal activity of the product on test objects.

The preparation of the following composition was used as the tested disinfectant: the mixture of quaternary ammonium compounds — 25%, glutar aldehyde — 11%, isopropanol, non-ionic surfactants.

The following viruses were used as a model to study the virucidal properties of the disinfectant:

— the epizootic virus of influenza A/chirianka/Dzhankoy/4-17-11/10 (H5N2) that was isolated from wild birds in the AR Crimea in 2010 by the staff of the National Scientific Center 'Institute of Experimental and Clinical Veterinary Medicine'. When infecting chicken embryos (CE), the 1.78 lg ELD_{50}/0.2 cm^3 was lethal. The virus A/chirianka/Dzhankoy/4-17-11/10 (H5N2) was deposited in the State Scientific and Control Institute of Biotechnology and Strains of Microorganisms (Kyiv), registration No. 383. The patent of Ukraine for the useful model No. 80546 was received for the virus A/chirianka/Dzhankoy/4-17-11/10 (H5N2). The extraembryonic liquid of the chicken embryos infected by the virus A/chirianka/Dzhankoy/4-17-11/10 (H5N2) was used as virus-containing liquid. The infective activity of the virus was 6.5 lg EID_{50}/0.2 cm^3, the titer of hemaglutinins was 1:512–1:1,024;

— the epizootic virus of Newcastle disease APMV-1/Pigeon/Doneck/3/2007 that was isolated from the wild birds in Donetsk in 2007 by the staff of the National Scientific Center 'Institute of Experimental and Clinical Veterinary Medicine'. The extraembryonic liquid of the chicken embryos infected by the virus APMV-1/Pigeon/Doneck/3/2007 was used as virus-containing liquid. The titer of hemaglutinins was 1:1,024–1:2,048.

9–10-day-old chicken embryos received from the hens having no antibodies to the viruses of Newcastle disease and avian influenza were used as a biological system for the virus indication. The incubation of the chicken embryos after the infection was carried out at the temperature 37.0 ± 0.5°C for 5 days.

The egg candling was conducted daily, twice a day. The recording was done if there had been the embryo mortality and the presence of hemaglutinin in the extraembryonic liquid that testified about the reproduction of Newcastle disease and avian influenza viruses in the embryos. The dead embryos as well as alive ones after 5 days of incubation were placed in the fridge at the temperature of 4–8°C for 12–18 hours and after that the autopsy was conducted. The extraembryonic liquid was taken from each embryo, the liquid was tested to detect the presence of hemagglutinating virus in the hemaglutination test with 1% suspension of the cock erythrocytes. The hemaglutination test was conducted in the in V-shaped plates by the generally accepted method. Not less than four embryos were used in each test.

The plates made from unpainted wood, metal, tile, textile (size 5×10 cm) were used to determine the disinfecting properties of the product.

All the experiments with the viruses of avian influenza and Newcastle disease were carried out in accordance with biosecurity rules in laminar box of the second class of protection that excludes the pathogen entering the environment. The permission for the work with the viruses of avian influenza and Newcastle disease is ISO 17025 (Certificate No. 2H1327).

Results and discussions. The influence of the work dilutions of the disinfectant under investigation had been previously studied in connection with the fact that the chicken embryos were used as the biological systems for the detection of the viruses of avian influenza and Newcastle disease.

Since chicken embryos were used as the biological system for detecting avian influenza and Newcastle disease viruses, we have previously studied the effect on them of working dilutions of the tested disinfectant.

For that the work dilutions of the drug in the concentrations of 0.1% and 0.5% were prepared. The sterile phosphate and salt buffer (pH 7.2–7.4) with antibiotics (penicillin 500 IU/cm^3, streptomycin 500 mkg/cm^3, nystatin 25 IU/cm^3) was used for the dilution. The work dilutions of the disinfectant were administered into allantois cavity of the 9–10-day-old chicken embryos at the dose of 0.2 cm^3. The observation of the embryos was conducted after the injection. The sterile phosphate and salt buffer (pH 7.2–7.4) with antibiotics was administered to the chicken embryos as a control. The obtained results are shown in Table 1.

Table 1 — Assessment of the effect of work dilution of disinfectant on chicken embryos (n = 3)

Work dilution		Quantity of dead embryos over time (hours) after infection				
		24	48	72	96	120
0.1	native	1	–	–	1	–
	10^{-1}	–	–	–	–	–
	10^{-2}	–	–	–	–	–
	10^{-3}	–	–	–	–	–
	10^{-4}	–	–	–	–	–
0.5	native	1	2	–	–	–
	10^{-1}	–	–	–	–	–
	10^{-2}	–	–	–	–	–
	10^{-3}	–	–	–	–	–
	10^{-4}	–	–	–	–	–
Control		–	–	–	–	–

Note: '–' — no cases of death.

During the study period (5 days), the death of embryos that were introduced native working dilutions of the disinfectant was observed. After 5 days of observation, all embryos were cooled under the temperature of 4–8°C for 12–18 hours and sectioned. The autopsy revealed growth and development retardation in embryos injected with native working dilutions of the drug and the absence of any abnormalities in embryos which were administered the drug in dilutions of 10^{-1} and higher (Fig.).

The data given above have proved that the tested disinfectant in 0.5% work dilution caused death and 0.1 % work dilution led to the retardation of development and death of the chicken embryos. When the preparation was used in the dilution of 10^{-1} and higher no deviations from the norm in the development of the embryos were detected, thus, they could be used in the further experiments as the biological system to assess the virulucudal properties of the disinfectant.

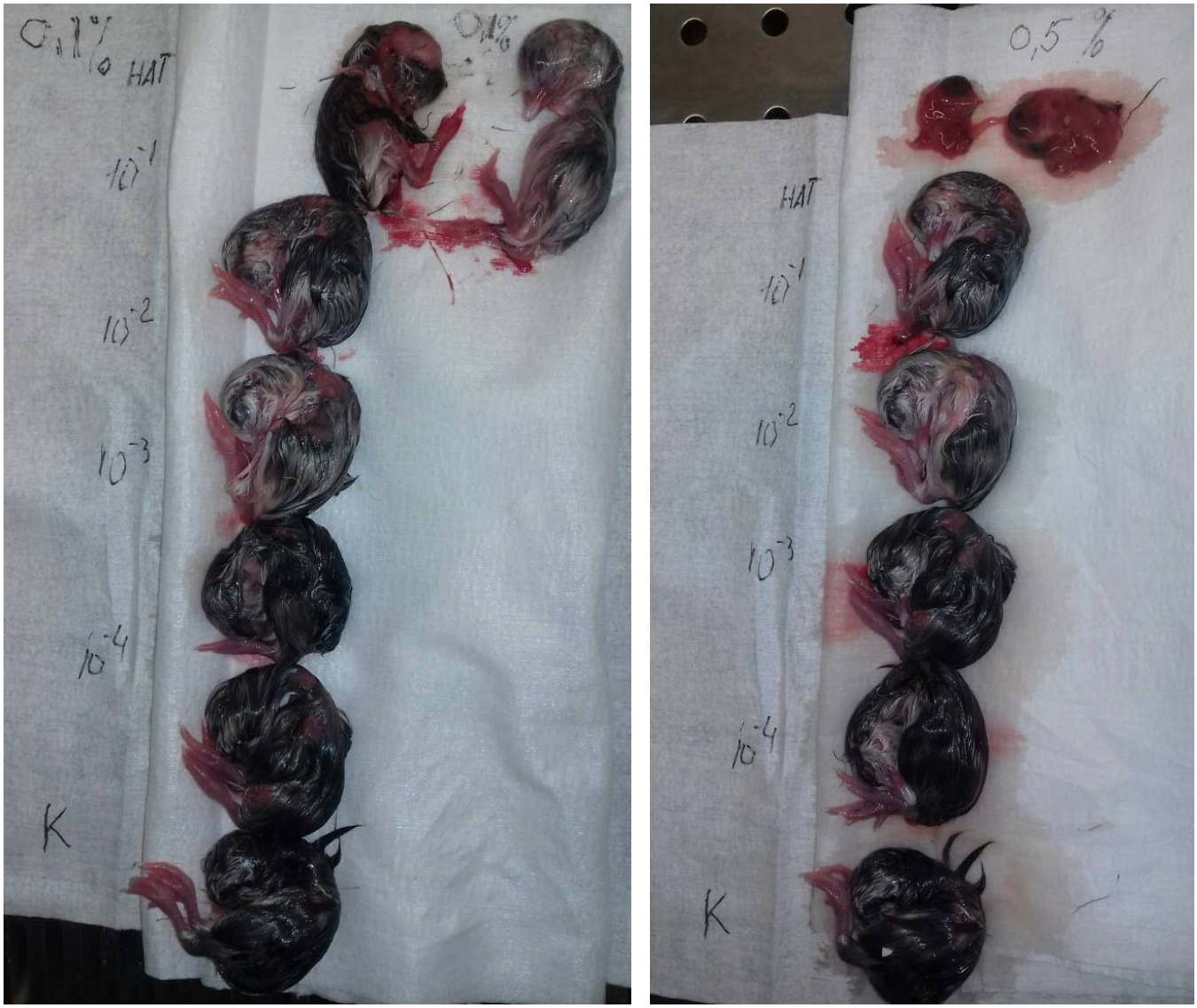

Figure. Chicken embryos after the administration of the work dilutions of the disinfectant

During the next stage of our research the determination of the virucidal activity of the disinfectant was carried out by the suspension method. This stage consisted of the preparation of the dilutions of the tested disinfectant, the verification of the titer of infective activity of avian influenza and Newcastle disease viruses, conducting of the main experiment and controls.

0.1 and 0.5% work dilutions of the disinfectant were used for the investigation. The preparation of the work dilutions was done on the sterile phosphate and salt buffer (pH 7.2–7.4) with antibiotics. The extraembryonic liquid with the viruses of Newcastle disease and highly pathogenic avian influenza was used in the dilution of 10^{-1}. Before the beginning of the experiment the infective titer of the viruses had been checked by the titration on the chicken embryos, the titer was calculated by Reed and Mench. The infection titer of avian influenza virus was 8.75 lg EID_{50}/0.2 cm^3, infection titer of Newcastle disease was 6.33 lg EID_{50}/0.2 cm^3.

The sterile penicillin bottles with the volume of 10 cm^3 were used for the experiment. The equal volumes of the extraembryonic liquid with the viruses of avian influenza and Newcastle disease in the dilution of 10^{-1} and the work dilutions of the disinfectant were mixed in the above bottles. The mixture was kept for 15, 30, 60, 90, 120 minutes under the room temperature. After finishing of each exposition, 1–2 ml of the mixture were taken and used to infect the chicken embryos. Each test was accompanied by the conducting of the controls: the positive control — the virus of highly pathogenic avian influenza with sterile water in the ratio 1:1, the negative one – sterile water. The infection of the embryos was carried out in accordance with the common method using disposable syringes for that. After the infection the embryos were observed for 4–5 days with daily ovoscopy. The recording of the results of the investigation was done by the embryo death and the presence of hemagglutinin in the extraembryonic liquid of the embryos. The death during the first day with the lack of hemaglutinin was thought to be non-specific. After the end of the above-mentioned period of observation all embryos that were alive were cooled and the section was conducted. The presence of viruses in the extraembryonic liquid was determined by hemaglutination test with 1% suspension of erythrocytes of the cock free from antibodies to the viruses of Newcastle disease and avian influenza (Tables 2 and 3).

As can be seen from Table 2, the disinfectant has virucidal properties to avian influenza virus (completely neutralizes the infectious properties of influenza virus) at concentrations not lower than 0.1% and exposure of not less than 30 minutes.

It can be seen from Table 3, that the disinfectant has virucidal properties to the virus of Newcastle disease (it completely neutralized the infective properties of the virus) when the concentration was not lower than 0.1% and the exposition was not less than 30 minutes.

After obtaining positive results in the studies by suspension method, further experiments were performed using test objects.

Thus, the investigation was carried out to determine the virucidal activity of the disinfectant on the surfaces that were contaminated by the viruses of avian influenza and Newcastle disease. For that previously cleaned, washed and sterilized test objects were used unpainted wood, metal, tile, textile with area 50 cm^2.

The concentrations of the disinfectant (0.1 and 0.5%), that had shown their effectiveness at the suspension method, were used for the investigation.

Table 2 — Determination of virucidal activity of the disinfectant to avian influenza virus by the suspension method (n = 4)

Concentration of disinfectant, %	Exposition, min	Quantity of chicken embryos		Results of hemagglutination test
		infected	died	
0.1	15	4	0	+ + – –
	30	4	0	– – – –
	60	4	0	– – – –
	90	4	0	– – – –
	120	4	0	– – – –
0.5	15	4	0	– – – –
	30	4	0	– – – –
	60	4	0	– – – –
	90	4	0	– – – –
	120	4	0	– – – –
Positive control	–	4	0	+ + + +
Negative control	–	4	0	– – – –

Table 3 — Determination of the virucidal activity of the disinfectant to Newcastle disease virus by the suspension method (n = 4)

Concentration of disinfectant, %	Exposition, min	Quantity of chicken embryos		Results of hemagglutination test
		infected	died	
0.1	15	4	0	+ + + –
	30	4	0	– – – –
	60	4	0	– – – –
	90	4	0	– – – –
	120	4	0	– – – –
0.5	15	4	0	– – – –
	30	4	0	– – – –
	60	4	0	– – – –
	90	4	0	+ + + +
	120	4	0	– – – –
Positive control	–	4	0	+ + + –
Negative control	–	4	0	– – – –

The necessary amount of the virus-containing liquid was put on the clean sterile surfaces (wood, metal, tile, textile) and evenly distributed on the whole surface with the help of a glass stick. After that, the test objects were kept horizontally for 1–2 hours at the room temperature for drying. Then the surfaces were irrigated with aerosol of approximately 20 cm^3 of the work solution of the disinfectant in such a way that all the surface of the test object was treated by the disinfectant. The exposition after irrigation was 30, 60, 90, and 120 minutes. After the end of each exposition the washouts from the surfaces were done by the sterile cotton swabs, then they were placed into the bottles with 1 cm^3 phosphate and salt buffer for 10 minutes and then they were shaken intensively and used for the infection of the chicken embryos by the methods described above. The washouts from the contaminated surfaces treated by sterile phosphate and salt buffer were used as the positive control. Sterile phosphate and salt buffer was used as a negative control.

The test object (textile) was prepared in the following way. The sterile clean pieces of the textile were saturated by the virus-containing liquid and they were kept at the room temperature for 1–2 hours for drying. After that the textile was completely dipped in the work dilution of the disinfectant in the concentrations mentioned above and it was kept there for 30, 60, 90, and 120 minutes. After the end of the exposition the textile was taken to the sterile phosphate and salt buffer for 10 minute, it was intensively shaken and used for the infection of the chicken embryos by the methods described above. The textile saturated by the virus and dipped into the sterile phosphate and salt buffer was used as the positive control. Sterile phosphate and salt buffer was used as a negative control.

After the infection the embryos were observed for 5 days with daily ovoscopy. The recording of the results of the investigation was done by the embryo death and the presence of hemagglutinin in the extraembryonic liquid of the embryos. The death during the first day with the lack of hemaglutinin was thought to be non-specific. After the end of the above-mentioned period of observation all the embryos that were alive were cooled and the section (autopsy) was conducted. The presence of viruses in the extraembryonic liquid was determined by the hemaglutination test with 1% suspension of erythrocytes of the cock free from antibodies to the viruses of Newcastle disease and avian influenza.

The results of the virucidal effect when disinfecting the surfaces are shown in Tables 4 and 5.

Thus, it has been found out (Table 4) that the work solution of the disinfectant in the concentrations of 0.1% and higher completely inactivated the infective properties of the viruses of avian influenza and Newcastle disease on all test objects 30 minutes after the treatment of the contaminated surface by irrigation. The viruses of avian influenza and Newcastle disease on the saturated textile were completely inactivated when dipping in the work solution of the disinfectant with the exposition of 30 minutes and more.

Table 4 — Results of determination of virucidal properties of the disinfectant in 0.1% work dilution with the use of test objects (n = 3)

Test object	Exposition, min	Quantity of chicken embryos		Results of hemagglutination test
		infected	died	
to avian influenza virus				
metal	30	3	–	– – –
	60	3	–	– – –
	90	3	1*	– – –
	120	3	1*	– – –
	control	2	1	+ –
tile	30	3	–	– – –
	60	3	–	– – –
	90	3	–	– – –
	120	3	–	– – –
	control	2	2 (from which 1*)	+ +
textile	30	3	–	– – –
	60	3	–	– – –
	90	3	–	– – –
	120	3	–	– – –
	control	2	1*	+ –
wood	30	3	–	– – –
	60	3	–	– – –
	90	3	–	– – –
	120	3	–	– – –
	control	2	1*	+ –
to Newcastle disease virus				
metal	30	3	1	– – –
	60	3	–	– – –
	90	3	1	– – –
	120	3	–	– – –
	control	2	2	+ +
tile	30	3	–	– – – –
	60	3	1	– – –
	90	3	–	– – –
	120	3	–	– – –
	control	2	1	+ +
textile	30	3	–	– – –
	60	3	–	– – –
	90	3	2*	– – –
	120	3	–	– – –
	control	2	2 (from which 1*)	+ +
wood	30	3	–	– – –
	60	3	–	– – –
	90	3	1	– – –
	120	3	–	– – –
	control	2	2	+ +

Note: * — non-specific death.

Table 5 — Results of determination of virucidal properties of the disinfectant in 0.5% work dilution with the use of test objects (n = 3)

Test object	Exposition, min	Quantity of chicken embryos		Results of hemagglutination test
		infected	died	
to avian influenza virus				
metal	30	3	–	– – –
	60	3	–	– – –
	90	3	1	– – –
	120	3	1	– – –
	control	2	1	+ –
tile	30	3	–	– – –
	60	3	–	– – –
	90	3	–	– – –
	120	3	–	– – –
	control	2	2 (from which 1*)	+ +
textile	30	3	–	– – –
	60	3	–	– – –
	90	3	–	– – –
	120	3	–	– – –
	control	2	1*	+ –
wood	30	3	1	– – –
	60	3	–	– – –
	90	3	–	– – –
	120	3	–	– – –
	control	2	1*	+ –
to Newcastle disease virus				
metal	30	3	–	– – –
	60	3	2*	– – –
	90	3	–	– – –
	120	3	1*	– – –
	control	2	2	+ +
tile	30	3	–	– – –
	60	3	–	– – –
	90	3	1*	– – –
	120	3	–	– – –
	control	2	1	+ +
textile	30	3	–	– – –
	60	3	–	– – –
	90	3	–	– – –
	120	3	1*	– – –
	control	2	2 (from which 1*)	+ +
wood	30	3	1	– – –
	60	3	–	– – –
	90	3	–	– – –
	120	3	–	– – –
	control	2	2	+ +

Note: * — non-specific death.

When testing the work dilution of the disinfectant in the concentration of 0.5 % (Table 5) it has been proved that the above concentration caused the inactivation of the viruses on the wood, tile, metal 30 minutes after the treatment.

The textile that had been contaminated by the viruses of avian influenza and Newcastle disease was disinfected during 30 minutes when dipping in 0.5% solution of the disinfectant.

Thus, as a result of the experiments conducted by the suspension method and with the help of test objects, the parameters of disinfection by the innovative disinfectant for the surfaces made from different materials and contaminated by the viruses of avian influenza and Newcastle disease were determined (Table 6).

Table 6 — The parameters of disinfection of different objects from viral contamination by the avian influenza virus with the help of the innovative disinfectant

Surface (material)	Concentration (not less than), %	Exposition (not less than), min	Disinfection method
metal	0.1	30	irrigation
tile	0.1	30	irrigation
wood	0.1	30	irrigation
textile	0.1	30	dipping

The results of the conducted research give the possibility to increase the arsenal of disinfectants that have high virucidal properties to the pathogens of the main infectious diseases of poultry.

Conclusions. The virucidal properties of the innovative disinfectant (the mixture of quaternary ammonium compounds — 25%, glutar aldehyde — 11%, isopropanol, non-ionic surfactants) to the viruses of avian influenza and Newcastle disease have been determined by the suspension method. It has been found that the above preparation completely inactivated the infective properties of the viruses when used in the concentration of 0.1%, with the interval of 30 minutes and in the concentration of 0.5% — with the interval of 15 minutes.

The preparation disinfected the test objects (wood, metal, tile, textile) that were contaminated by the pathogenic agents of avian influenza and Newcastle disease in the concentration of 0.1%, the exposition of 30 minutes.

The above disinfectant can be used for conducting the preventive and urgent disinfection of the facilities in the poultry farms taking into consideration the parameters given above.

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Contents

Part 1. Veterinary medicine

Part 2. Biotechnology and genetics

Part 3. Biosafety