ISSN 2411-0388

NATIONAL ACADEMY OF AGRARIAN
SCIENCES OF UKRAINE

NATIONAL SCIENTIFIC CENTER
**'INSTITUTE OF EXPERIMENTAL
AND CLINICAL VETERINARY MEDICINE'**

JOURNAL FOR VETERINARY MEDICINE, BIOTECHNOLOGY AND BIOSAFETY

Volume 8
Issue 3–4

KHARKIV
2022

The Journal for Veterinary Medicine, Biotechnology and Biosafety is included **in the 'List of Scientific Special Serial Publications' of Ukraine (category 'B', specialities: 091** — Biology, 211 — Veterinary Medicine, 212 — Veterinary Hygiene, Sanitation and Expertise) that can publish the results of Ph.D. and Dr.Habil. theses in biological and veterinary sciences (orders of the Ministry of Education and Science of Ukraine: № 1328, December 21, 2015**;** № 515, May 16, 2016; № 886, July 2, 2020)

Materials approved for publication and to spread via the Internet by the Scientific Council of the National Scientific **Center 'Institute of Experimental and Clinical Veterinary Medicine'** (protocol No. 14 of December 22, 2022)

The full text of articles available at jvmbbs.kharkov.ua. JVMBBS covered in the abstract and citation databases Google Scholar (scholar.google.com), Index Copernicus (indexcopernicus.com), and CrossRef (crossref.org)

Editorial Board Address:
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Certificate of state registration:
KB No. 21398-11198P of June 25, 2015

Part 1. Veterinary medicine

UDC 619:616.98-036.22:579.843.94:636.52/.58(477) DOI 10.36016/JVMBBS-2022-8-3-4-1

STUDY OF THE PATHOGENIC PROPERTIES OF *AVIBACTERIUM PARAGALLINARUM* CULTURES ISOLATED IN 2019–2020

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Summary. Both viruses and bacteria, including *Avibacterium paragallinarum*, formerly known as *Haemophilus paragallinarum*, can be etiological agents of respiratory infections of birds. Cultural-morphological and molecular-biological studies established that three isolates selected during 2019–2020 from pathological material (swabs from the subocular sinuses) of 42–180 days old birds (No. 1 — SS 6/20, serotype A; No. 2 — SS 7/20, serotype B; No. 3 — SS 8/20, serotype C) belonged to the species *A. paragallinarum* and they formed a heterogeneous group. During the study of the virulence of isolates on birds, it was found that it varies: *A. paragallinarum* SS 6/20 is virulent (the average value of the sum of points is from 0.5 to 0.7); *A. paragallinarum* SS 7/20 is low virulent (the average value of the sum of points is from 0.2 to 0.3); *A. paragallinarum* SS 8/20 is virulent (the average value of the sum of points is from 0.8 to 0.9). Also, isolates were heterogeneous in terms of pathogenicity. The pathogen *A. paragallinarum*, SS 7/20 had the lowest pathogenicity, while when infected with *A. paragallinarum* isolates, SS 6/20 and *A. paragallinarum*, SS 8/20, the morbidity of birds was 80–100%

Keywords: avian infectious rhinitis, avian infectious coryza, virulence

Introduction. Infectious rhinitis (poultry hemophilosis) is an acute enzootic highly infectious disease of the upper respiratory tract of poultry, primarily chickens, characterized by catarrhal inflammation of the mucous membranes of the nasal cavity, conjunctiva and sinuses, as well as subcutaneous swelling of the head and in rare cases — pneumonia.

This disease spreads horizontally. The source of infectious rhinitis is a sick and recovered bird, in the body of which bacteria can persist for 6–12 months (Baydevlyatov et al., 1980; Calnek, 2003; Korniienko et al., 2012; Orlov, 1971; Orlov and Prokof'eva, 1962). Information on the carrier of *A. paragallinarum* by wild birds has been published, which makes it a potential reservoir of infection (Calnek, 2003).

Birds of all ages are susceptible to infectious rhinitis, but especially chickens over 4 days old. It is believed that infectious rhinitis is not a systemic disease and does not cause high mortality in susceptible birds, but during acute outbreaks, flock losses can reach 10%. Economic losses are also manifested in the reduction of egg laying in hens by up to 40%, especially at the peak of productivity, and growth retardation in young birds (Korniienko et al., 2012).

In young birds, the disease, as a rule, begins with nonspecific clinical signs, such as depression, growth retardation, drowsiness ('sleeping bird' syndrome). Older chickens have sinusitis, hemorrhagic conjunctivitis, serous and/or serous-fibrinous rhinitis. As the disease progresses, a syndrome a syndrome occurs, which is often seen in avian metapneumovirus infection, better known as SHS (or 'swollen head syndrome'); it causes blindness. Infectious rhinitis is also associated with such pathological changes as fibrinous lesions of the submandibular area, one- or two-sided aerosacculitis, septic lesions of the liver and kidneys, less often, as already mentioned, with pneumonia.

According to antigenic properties, strains of *A. paragallinarum* are classified following two main schemes proposed by Page (1962) and Kume et al. (1983), Morales-Erasto et al. (2014). The scheme of Blackall et al. (1990), in which all strains of the causative agent of infectious rhinitis are divided into three serotypes: A, B, and C, is the most widespread. These serotypes do not have cross-protection during poultry immunization.

The work is aimed to study the cultural and morphological characteristics of epizootically relevant strains (isolate No. 1 — SS 6/20, serotype A; isolate No. 2 — SS 7/20, serotype B; isolate No. 3 — SS 8/20, serotype C) *A. paragallinarum*, which were isolated in recent years (2019–2020), as well as to determine their pathogenicity and virulence in susceptible birds with further use of the knowledge gained for the construction of an inactivated vaccine and as control strains to determine the immunogenicity of a prophylactic biological preparation.

Materials and methods. In order to definitively identify *A. paragallinarum* isolates (SS 6/20, serotype A;

SS 7/20, serotype B; SS 8/20, serotype C), which were isolated in 2019–2020 from pathological material, additionally a number of laboratory studies of cultural and biochemical properties were carried out. We also took into account the ability of cultures to grow on nutrient media without blood serum under the increased content of carbon dioxide in the atmosphere, to produce various metabolites and enzymes, to break down carbohydrates.

Biochemical properties were determined in *A. paragallinarum* cultures grown on a dense nutrient medium after 20–24 hours of incubation at 37 °C by culturing in the nutrient medium 'Broth base with phenol red M 054', produced by 'Himedia'. Oxidase activity was determined using a commercial kit following the instructions for use. The absence of catalase production was checked using a 3% hydrogen peroxide solution.

The next stage of laboratory research of selected isolates was to determine their pathogenicity for birds.

In a series of experiments, a 20-hour broth culture of the three *A. paragallinarum* isolates was used, in which the number of viable cells was determined by plating serial dilutions on a dense nutrient medium — Columbia serum agar with the addition of NAD. 8-week-old broiler chickens of the cross 'KOBB-500' were used as test objects. Each experimental group consisted of five birds, the experiment was carried out in two repetitions.

The experiment on virulence (clinical manifestation of infection) we used the methodology proposed by Soriano et al. (2004). The presence and degree of the upper respiratory tract damage in the infected poultry was assessed following to the scoring system:

0 — absence of clinical signs;

1 — scanty discharge of exudate from the nasal passages and/or slight swelling of the infraorbital sinuses;

2 — moderate discharge of exudate from the nasal passages and/or moderate swelling of the area of the infraorbital sinuses;

3 — profuse nasal discharge of exudate and/or severe swelling of the infraorbital sinuses;

4 — profuse nasal discharge of exudate and pronounced swelling of the infraorbital sinuses, wheezing.

Clinical signs were recorded daily in each bird separately. 5 days after infection, the sum of points for each group was determined, which was divided by the total number of infected birds.

Criteria for assessing virulence:

≤ 0.5 — low virulent;

0.5–0.9 — pathogenic;

≥ 1.0 — highly virulent.

21 days after experimental infection, all chickens were subjected to forced slaughter. After the post-mortem examination of poultry, bacteriological analysis of the infraorbital sinuses' contents was carried out to re-isolate the causative agent for the confirmation of the disease specificity.

The pathogenicity of the isolates was established by determining the minimum infectious dose that would ensure the disease of 80–100% of the birds in the experiment (Anjaneya et al., 2013).

Results. According to the previously obtained results from the study of cultural and morphological properties and PCR of three isolates of chicken hemophilosis (No. 1 — SS 6/20, serotype A; No. 2 — SS 7/20, serotype B; No. 3 — SS 8/20, serotype C) it was established that all cultures belong to the species *A. paragallinarum*.

The characteristics of the biochemical activity of these isolates are presented in Table 1, where we can see that they are also a homogeneous group in terms of cultural properties and enzymatic activity.

Table 1 — Cultural properties of *A. paragallinarum* isolates

Isolate	Galactose	Glucose	Mannitol	Sorbitol	Saccharose	Trehalose	Catalase	Oxidase	Indole	H_2S	Nitrate recovery	Hemolysis on blood agar	NAD dependence	Blood serum requirement
SS 6/20, serotype A	–	+	+	+	+	–	–	–	–	–	+	–	+	+
SS 7/20, serotype B	–	+	+	+	+	–	–	–	–	–	+	–	+	+
SS 8/20, serotype C	–	+	+	+	+	–	–	–	–	–	+	–	+	+

The characteristic properties of the bacteria were: the ability to reduce nitrates to nitrites, the absence of indole, hydrogen sulfide, catalase and oxidase production.

The saccharolytic activity of the selected isolates also did not differ. Thus, all isolates fermented glucose and sucrose, but not lactose, trehalose and galactose.

It is well known that there are various types of pathogenicity factors of infectious diseases agents, including hemophilosis of birds, from the presence of receptors for attachment to cells, properties regarding colonization (distribution throughout the body) and invasion (penetration into cells), negative impact on the body's immune response (binding antibodies with pathogen proteins) and reproduction of toxins. Therefore, to characterize different degrees of pathogenicity and its quantitative expression, the term virulence (degree of pathogenicity) is used.

In our study, the assessment of *A. paragallinarum* (SS 6/20, serotype A; SS 7/20, serotype B; SS 8/20, serotype C) virulence was performed following the

method of Soriano et al. (2004). Poultry was infected intranasally with a 20-hour daily broth culture of isolates in a dose of 0.5 cm^3, with a content of at least 10^8 CFU of the pathogen. The results of these studies on broiler chickens are presented in Table 2.

Table 2 — Virulence of *A. paragallinarum* isolates for 'KOBB-500' 8-week-old broiler chickens

Isolate	Time after infection, days						Average value
	1	2	3	4	5	6	
	Sum of points by groups						
SS 6/20, serotype A (I experiment)	0.5	0.6	0.8	0.8	0.6	0.4	0.5
SS 6/20, serotype A (II experiment)	0.6	0.8	1.0	0.8	0.5	0.4	0.7
SS 7/20, serotype B (I experiment)	0	0	0.2	0.4	0.4	0.4	0.2
SS 7/20, serotype B (II experiment)	0	0.2	0.4	0.5	0.5	0.4	0.3
SS 8/20, serotype C (I experiment)	0.6	0.8	0.8	1.0	0.8	0.8	0.8
SS 8/20, serotype C (II experiment)	0.6	0.8	1.0	1.0	1.0	0.8	0.9

As can be seen from the results of laboratory studies, the virulence of *A. paragallinarum* isolates is different and the average value of the sum of points is: SS 6/20, serotype A — from 0.5 to 0.7; SS 7/20, serotype B — from 0.2 to 0.3; SS 8/20, serotype C — from 0.8 to 0.9.

The results of experimental infection of poultry are presented in Table 3. Different infecting doses of isolates were used to infect poultry. The doses depended on the concentration of viable cells and varied from 4.78 ± 0.13 to $6.25 \pm 0.32 \times 10^8$ CFU.

The first clinical signs of the disease in poultry were observed 24–48 hours after infection. Symptoms were manifested in the form of liquid discharge from the nostrils and slight one- or two-sided swelling of the infraorbital sinuses.

Later, unilateral or bilateral catarrhal conjunctivitis developed in some birds, sometimes fibrin appeared in the exudate, which led to swelling of the eyelids and narrowing of the eye slit. When the infection was localized in the deeper parts of the respiratory tract, breathing was accompanied by wheezing in some chickens. In our studies, the period of clinical manifestation of the disease was 3–6 days.

During the analysis of the obtained results, it was established that isolates of the causative agent of chicken infectious rhinitis are heterogeneous in terms of pathogenicity. The pathogen *A. paragallinarum*, SS 7/20, serotype B had the lowest pathogenicity, at the same time in the case of infection with isolates *A. paragallinarum*, SS 6/20, serotype A and *A. paragallinarum*, SS 8/20, serotype C, the morbidity of poultry was 80–100%. 21 days after experimental infection, the pathogen was re-isolated from the contents of the infraorbital sinuses in most birds, regardless of the presence and severity of clinical signs during the disease period.

Table 3 — Study of the pathogenic properties of *A. paragallinarum* isolates for 8-week-old broiler chickens, cross KOB-500

Strain name	Infectious dose, $\times 10^8$ CFU	Number of birds		Morbidity, %	Reisolation of the pathogen, %
		infected	with clinical manifestation of infection		
SS 6/20, serotype A (experiment I)	4.78±0.13	5	4	80	60
SS 6/20, serotype A (experiment II)	5.13±0.16	5	4	80	80
SS 7/20, serotype B (experiment I)	5.60±0.19	5	2	40	60
SS 7/20, serotype B (experiment II)	5.87±0.23	5	3	60	80
SS 8/20, serotype C (experiment I)	6.10±0.25	5	4	80	80
SS 8/20, serotype C (experiment II)	6.25±0.32	5	5	100	100

Discussion. Among the infectious diseases of poultry, chicken hemophilus, the causative agents of which are bacteria *Avibacteruim paragallinarum*, formerly known as *Haemophilus paragallinarum* (Calnek, 2003; Blackall and Soriano-Vargas, 2020; Blackall et al., 2005), is one of the main problems for commercial poultry farming worldwide (Blackall et al., 2005; Blackall et al., 1997; Kelser, 1997; Blackall and Yamamoto, 1998; Poernomo et al., 2000).

According to preliminary cultural and morphological studies and PCR, it has been established that three isolates (No. 1 — SS 6/20, serotype A; No. 2 — SS 7/20, serotype B; No. 3 — SS 8/20, serotype C) isolated during 2019–2020 from the pathological material of birds aged 42–180 days with clinical signs of the disease (rhinitis, fibrinous inflammation of the mucous membranes of the sinuses), belong to the species *A. paragallinarum* and they form a heterogeneous group. Thus, the cultures were capsule-forming short rods or coccobacilli that required V-growth factor.

In addition, all *A. paragallinarum* isolates were NAD-dependent, although in the special literature there are reports on the NAD-independent isolates of the pathogen (Mouahid et al, 1992). The dependence of the cultivation of isolates on the presence of blood serum in the nutrient medium turned out to be absolute, which is

consistent with the results of research by other authors (Calnek, 2003; Deshmukh, 2015). Growth of *A. paragallinarum* on serum-free medium could not be obtained, even when it contained the optimal amount of V-factor. In addition, a number of foreign researchers claim that the cultivation of the causative agent of infectious rhinitis of chickens is possible only in the presence of an increased content of carbon dioxide (8–10%) in the atmosphere (Calnek, 2003; Deshmukh, 2015). However, in our experiments, the isolates did not show such a dependence. The morphology and size of the colonies obtained under the conditions of a normal atmosphere did not differ in any way from cultures with an increased content of carbon dioxide. The variability of signs was observed in the ratio of mannitol and mannose, as evidenced by the results of other authors (Blackall et al., 2005; Deshmukh, 2015).

In addition, according to literature data, it is known that apathogenic species of hemophilic bacteria *A. avium, A. volantium* do not require blood serum, produce catalase, ferment trehalose and galactose (Patil et al., 2017).

The virulence of the isolates was determined by intranasal administration of bacterial suspension of the pathogen to susceptible broiler chickens. There are many reports on these studies in the literature (Matsumoto and Yamamoto, 1975; Rimler et al., 1977; Bragg, 2002a; Bragg, 2002b).

Thus, in our studies, the virulence of experimental *A. paragallinarum* isolates varied. A high average score was noted using isolates SS 8/20 (serotype C) — from 0.8 to 0.9 (virulent) and SS 6/20 (serotype A) — from 0.5 to 0.7 (virulent). In turn, isolate SS 7/20 (serotype B) had the lowest value — from 0.2 to 0.3 (low virulence).

It should also be noted that clinical signs of the disease in the infected poultry already began to be noted on the first or second day (discharge from the nostrils, slight swelling of the infraorbital sinuses). In the future, the clinical manifestations of the disease progressed, which led to swelling of the eyelids and narrowing of the eye slit. In addition, in some chickens, breathing was accompanied by wheezing.

In general, the investigated *A. paragallinarum* isolates were not homogeneous in terms of pathogenicity. Thus, isolate SS 8/20 (serotype C) demonstrated high pathogenicity in relation to chickens, as well as high rates of re-isolation (80–100%). According to the literature, such a high pathogenicity of the SS 8/20 isolate may be related to its high reproductive capacity (Bragg, 2002b). In turn, SS 6/20 and SS 7/20 isolates (80% and 40–60%) were somewhat inferior to SS 8/20 in terms of pathogenicity; in terms of reproducibility, they were also lower than SS 8/20, but the same between themselves (60–80%).

Conclusions. 1. Based on the results of cultural, morphological and biochemical properties, three isolates of avian hemophilosis selected from clinically sick birds (SS 6/20, serotype A; SS 7/20, serotype B; SS 8/20, serotype C) were assigned to the species *A. paragallinarum*. Cultures are gram-negative capsule-forming short rods or coccobacilli, which require the presence of V-factor and blood serum during cultivation; they reduce nitrates to nitrites, ferment glucose and sucrose, but not lactose, trehalose and galactose; the absence of indole, hydrogen sulfide, catalase, and oxidase production is noted.

2. It has been established that the virulence of *A. paragallinarum* isolates is different and is: *A. paragallinarum* SS 6/20, serotype A is virulent (the average value of the sum of points is from 0.5 to 0.7); *A. paragallinarum* SS 7/20, serotype B is low virulent (the average value of the sum of points is from 0.2 to 0.3); *A. paragallinarum* SS 8/20, serotype C is virulent (the average value of the sum of points is from 0.8 to 0.9).

3. *A. paragallinarum* isolates are heterogeneous in pathogenicity. The pathogen *A. paragallinarum*, SS 7/20, serotype B had the lowest pathogenicity, while in the case of infection with *A. paragallinarum* isolates, SS 6/20, serotype A and *A. paragallinarum,* SS 8/20, serotype C, the morbidity of birds was 80–100%. The development of infection was proven by reisolation of the pathogen 21 days after experimental infection from the contents of the infraorbital sinuses, regardless of the presence and severity of clinical signs.

4. Taking into account the pathogenic characteristics and the degree of virulence, these isolates are available for obtaining a highly specific and active antigen, production of experimental series of inactivated vaccines and conducting immunogenicity control.

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UDC 619:638.15-084:602.3:579.864.1:579.873.13 DOI 10.36016/JVMBBS-2022-8-3-4-2

FACTORS OF NON-SPECIFIC RESISTANCE OF BEE HEMOLYMPH WHEN FEEDING PROBIOTIC DRUG 'BILAKT'

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Summary. The paper presents the results of the feeding the 'Bilakt' probiotic on factors of non-specific resistance in bees. Hemolymph samples were taken on the 7th, 14th, and 21st days, and the activity of lysozyme and phagocytosis, bactericidal activity were determined. According to the research results, the lysozyme activity in the hemolymph of 3–5-day-old larvae and nurse bees of the experimental groups after 21 days was reliably 1.5 times higher than the control group and before feeding. The increase in the hemolymph bactericidal factor in 3–5-day-old larvae, and in nurse bees — threefold on the 21st day after the end of feeding with 'Bilakt' drug. Phagocytic activity before the beginning of the experiment in the hemolymph of 3–5-day-old larvae was 39.5%, on the 1st day after the end of feeding with 'Bilakt' it increased and exceeded this indicator by 29%. The phagocytic index before feeding was 2.04 ± 0.11. On the 1st day after the end of feeding, it increased by 18.4%, respectively. The phagocytic activity of hemolymph of nurse bees was 45.8%. Accordingly, the phagocytic index was 2.2 ± 0.12. On the 1st day after the end of 'Bilakt' feeding, phagocytic activity increased by 40.9%, the phagocytic index was 3.24 ± 0.1, which was 32.1% higher than the initial level. Research results indicate that the use of 'Bilakt' helps to improve the general physiological condition of sick bees by stimulating the cellular and humoral mechanisms of protection of insects from pathogens, i.e. increasing the non-specific protective properties of both the body of 3–5-day-old larvae and bee adults

Keywords: prevention, bactericidal activity, phagocytic activity

Introduction. Among the urgent tasks of veterinary support of beekeeping, the use of ecologically safe means for bee disease prevention, harmonized with EU requirements, is gaining importance. The search for effective biological agents that do not harm the bees and the quality of the products produced by them is the primary task of a veterinary specialist (Bugera, 2008; Fedoruk and Kovalchuk, 2013; Tran et. al., 2013).

In this regard, the use of probiotics deserves special attention in the system of bee disease prevention (Dvylyuk, 2013; Collins and Gibson, 1999; Galatiuk et. al, 2020). In honeybees, the gut contains a microbiome composed of various bacterial taxa that influence the stimulation of immune and metabolic pathways, digestion or detoxification of food, and defense against pathogens and parasites. Stressors, including toxins and poor nutrition, disrupt the microbiome and increase susceptibility to opportunistic pathogens (Motta et. al, 2022; Anderson et. al, 2013; Audisio and Benítez-Ahrendts, 2011; Corby-Harris et.al, 2014; Endo and Salminen, 2013).

The participation of the bee organism in limiting the spread of infectious agents depends not only on the efficiency of the immune response. It is also determined by non-specific resistance factors, which are functionally based on phagocytosis, stimulation of humoral defense mechanisms and are the most effective first barrier in the fight against the causative agent of the disease. Among humoral factors of natural resistance, an important role is played by the enzyme lysozyme, which, being adsorbed on the mucopeptide cell wall of a microorganism, splits them (Fedoruk et al., 2009). Lysozyme increases its activity in a short period of time after the disease-causing agent enters the body of insects and is one of the starters of other protective factors. Lysozyme is a relatively small protein molecule (about 15 kDa). Its concentration in the hemolymph of larvae and adults ranges from 5 to 25 mg/ml. The antibacterial response is carried out by increasing the activity of lysozyme. Thus, the life products of lactobacilli, representatives of the beneficial microflora of the bees' intestines, contribute to the increase of a complex of factors of non-specific resistance: the content of lysozyme, the phagocytic and bactericidal activity of the hemolymph of bees (Fedoruk et al., 2009; Glynski and Jarosz, 1993; Boman, 1982).

The purpose of the study. In this regard, this work aimed to determine the effect of the probiotic 'Bilakt', which includes lactic acid bacteria (LAB) from the genus *Lactobacillus*, as well as bacteria from the genus *Bifidobacterium*, on factors of non-specific resistance of bee hemolymph: bactericidal and phagocytic activity in general, and in particular lysozyme activity.

Materials and methods. The experiments were conducted in the Sector of Bee Disease Study and the research apiary of the National Scientific Center 'Institute of Experimental and Clinical Veterinary Medicine'. Research period: 3rd decade of May. One experimental and one control group of similar bee families-analogs (three in each) were formed. By the time the control and experimental groups were formed, the bee families had

4.0 kg of bees, 5.0 kg of fodder honey, a two-year-old queen, sealed brood on 3 frames (240 squares) and 2 frames with open brood.

The first group of bee families was the control. For feeding, these bee colonies were given sugar syrup (1:2), prepared in boiled water, at the rate of 50 ml per seam of bees (on average 2,000 adults), with an interval of 2 days, 4 times, using a feeder. The second group was an experimental one, feeding was carried out at the same time as in the control group, with the same frequency, with sugar syrup, but with the addition of 'Bilakt' at the rate of 9 cm^3 per 2.5 liters of syrup. 'Bilakt' drug contains microorganisms *Lactobacillus plantarum* and *Bifidobacterium* sp. in the amount of 1×10^8 microbial cells/cm^3, as well as the nutrient medium on which they were cultivated. The total duration of the experiment was 30 days from the moment of the start of feeding.

Bee viability monitoring (death, food activity, behavior) was mainly carried out visually. From each group of bees, 100–150 specimens from frames with open brood with the largest number of 5–15-day-old nurse bees and 3–5-day-old larvae were selected. Selection and examination of collective samples of hemolymph from each group of bees was carried out before the beginning of feeding the experimental concentrations, as well as 7, 14, and 21 days after the end of 'Bilakt' feeding.

Determination of lysozyme activity in hemolymph was carried out by the turbidimetric method. The *Micrococcus lysodeikticus* culture was prepared in a phosphate buffer, the collected samples of hemolymph were diluted 10 times with physiological solution. The activity of lysozyme in the hemolymph sample was calculated according to the calibration curve in μg/ml (Labynskaya, 1978).

The bactericidal activity of hemolymph was studied by the method of diffusion in agar (Labynskaya, 1978). Pathogenic for bees microorganisms were used as test cultures — foulbrood causative agents: American (*Paenibacillus larvae*), European (*Melissococcus pluton*), para foulbrood (*Paenibacillus alvei*). A culture of microorganisms at a concentration of 1 billion cells/cm^3 was placed on the surface of the agar in a Petri dish. The dishes were kept in a thermostat at a temperature of 37 °C for 2 hours. As a marker, wells with a diameter of 3–4 mm were made, into which samples of hemolymph were introduced. The results were calculated after 24, 48, 72 hours (Labynskaya, 1978).

The indicator of phagocytosis activity of hemolymph cells was determined according to the Berman method (Labynskaya, 1978). To assess completed phagocytosis, we determined the number of cells that successfully completed the process (including fragments of destroyed cells) per 100 cells involved in the phagocytosis process.

We processed the results of the experimental studies statistically using the MS Excel 2010 computer program. To determine the arithmetic mean (M), its error (m), and the level of probability (p), we referred to the Student's *t*-test table by Melnychenko et al. (2006).

This research adheres to bioethical norms and complies with various guidelines. Specifically, it was conducted in accordance with the 'European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes' (CE, 1986) and Council Directive 2010/63/EU (CEC, 2010), Art. 26 of the Law of Ukraine No. 3447-IV of 21.02.2006 'About protection of animals from cruel treatment' (VRU, 2006).

Results and discussion. In the process of studying the effect of 'Bilakt' feeding on the body of bees, it was established that the hemolymph lysozyme activity indicators in the experimental group reliably increased in all individuals (Tabs 1, 2).

Table 1 — Hemolymph lysozyme activity of 3–5-day-old larvae (n = 30)

Bee groups	Selection period, day	Lysozyme activity, μg/ml
Control	Before feeding	42.20 ± 1.52
	7	41.97 ± 0.03
	14	41.67 ± 0.39
	21	41.83 ± 0.37
Experimental	Before feeding	48.39 ± 1.92
	7	64.00 ± 1.00*
	14	60.70 ± 4.11*
	21	64.00 ± 4.08*

Note: * — $p < 0.05$ reliably the indicators of the control group and prior to the start of the feeding process.

Table 2 — Hemolymph lysozyme activity of nurse bees (n = 30)

Bee groups	Selection period, day	Lysozyme activity, μg/ml
Control	Before feeding	44.80 ± 1.90
	7	44.43 ± 0.47
	14	43.29 ± 0.89
	21	43.60 ± 1.30
Experimental	Before feeding	43.30 ± 0.79
	7	62.59 ± 2.40*
	14	63.18 ± 2.40*
	21	66.51 ± 3.10*

Note: * — $p < 0.05$ reliably the indicators of the control group and prior to the start of the feeding process.

Based on the information provided in Table 1, it appears that the activity of lysozyme in the hemolymph of 3–5-day-old larvae in the experimental group increased by reliably 1.5 times after 7 days of consuming a mixture of 'Bilakt' and sugar syrup. This increase was also observed to be 1.4 times higher after 14 days and

1.5 times higher after 21 days, compared to the control group and the pre-feeding period.

According to the information presented in Table 2, it appears that the nurse bees in the experimental group exhibited a hemolymph lysozyme activity level that was reliably 1.4 times higher after being fed a mixture of 'Bilakt' and sugar syrup for 7 and 14 days, and 1.5 times higher after 21 days, in comparison to the control group and the pre-feeding period.

In the experimental group, it appears that the activity of lysozyme in the hemolymph of 3–5-day-old larvae increased by 34% after 7 days of not feeding, followed by a 31.4% increase after 14 days and a 34.7% increase after 21 days. Nurse bees also showed an increase in lysozyme activity, with a 29.1% increase on the 7th day, 31.5% — on the 14th day, and 34.5% — on the 21st day. However, there was no increase in lysozyme activity observed in bees from the control groups during the experiment.

The bactericidal activity of bee hemolymph was determined in the samples taken before the start of the experiment and 21 days after the end of feeding, it was established that the experimental parameters reliably increased in all individuals (Table 3).

Table 3 — Bactericidal activity of hemolymph of bees

Bee groups (n=30)	Bactericidal factor, h					
	P. larvae		*M. pluton*		*P. alvei*	
	larvae	nurse bees	larvae	nurse bees	larvae	nurse bees
Pre-feeding period	6	4	6	4	6	4
Control	6	4	6	4	6	4
Experimental, 21st day	12	12	12	12	12	12

From the data in Table 3, it can be seen that the bactericidal factor in the hemolymph of 3–5-day-old larvae doubled, and in nurse bees, it tripled on the 21st day after the end of feeding with 'Bilakt'.

Indicators of phagocytic activity of hemocytes of the hemolymph of 3–5-day-old larvae changed after feeding 'Bilakt'. The results are presented in Table 4.

From the data in Table 4, it can be seen that the phagocytic activity before the beginning of the experiment was 39.5%, on the 1st day after the end of feeding with 'Bilakt', it increased and exceeded this indicator by 29%, on the 7th day — 30.5%, on the 14th day — 27.8%, and on the 21st day — 27%.

The phagocytic index before feeding was 2.04 ± 0.11, on the 1st day after the end of feeding it increased by 18.4%, on the 7th, 14th, and 21st days it decreased by 2.0%, 2,8%, and 4.0%, respectively.

The dynamics of indicators of phagocytic activity and the phagocytic index of the hemolymph of nurse bees are presented in Table 5.

Table 4 — Indicators of phagocytic activity of hemolymph of 3–5-day-old larvae (n=30)

Period	Indicators				
	Total number of hemocytes, pcs.	Hemocytes that phagocytized microbes, pcs.	Number of microbial cells, pcs.	Phagocytic activity,%	Phagocytic index
Pre-feeding	253	100	204	39.5	2.04 ± 0.11
After feeding with the drug 'Bilakt' after days:					
1	180	100	250	55.6	2.50 ± 0.14
7	176	100	240	56.8	2.45 ± 0.14
14	183	100	245	54.6	2.43 ± 0.12
21	185	100	243	54.0	2.40 ± 0.12

Table 5 — Indicators of phagocytic activity of the hemolymph of nurse bees (n = 30)

Period	Indicators				
	Total number of hemocytes, pcs.	Hemocytes that phagocytized microbes, pcs.	Number of microbial cells, pcs.	Phagocytic activity,%	Phagocytic index
Pre-feeding	218	100	220	45.8	2.2 ± 0.12
After feeding with the drug 'Bilakt' after days:					
1	129	100	324	77.5	3.24 ± 0.10
7	133	100	307	75.2	3.07 ± 0.11
14	150	100	290	66.7	2.90 ± 0.12
21	175	100	282	57.1	2.82 ± 0.12

At the beginning of the experiment, phagocytic activity was 45.8%. Accordingly, the phagocytic index was 2.2 ± 0.12. On the 1st day after the end of 'Bilakt' feeding, phagocytic activity increased by 40.9%, the phagocytic index was 3.24 ± 0.1, which is 32.1% higher than the initial level. On the 21st day, these indicators decreased by 26.3% and 13%, respectively.

Our research findings suggest that 'Bilakt' positively impacts the overall physiological state of ill bees by enhancing their cellular and humoral defense mechanisms against pathogens, thereby in an increase in the non-specific protective features of both adult bees and 3–5-day-old larvae.

Conclusions. It was established that the use of the drug 'Bilakt' contributed to the stimulation of non-specific resistance of 3–5-day-old larvae and nurse bees. According to the research results, the activity of lysozyme in the hemolymph of 3–5-day-old larvae and nurse bees

of the experimental groups after 21 days was reliably 1.5 times higher compared to the control group and the pre-feeding period.

Bactericidal factor of the hemolymph of 3–5-day-old larvae doubled, and in nurse bees — tripled on the 21st day after the end of feeding with 'Bilakt'. Phagocytic activity before the beginning of the experiment in the hemolymph of 3–5-day-old larvae was 39.5%, on the 1st day after the end of feeding with 'Bilakt', it increased and exceeded this indicator by 29%.

The phagocytic index before feeding was 2.04 ± 0.11, on the 1st day after the end of feeding it increased by 18.4%, respectively. The phagocytic activity of hemolymph of nurse bees was 45.8%. Accordingly, the phagocytic index was 2.2 ± 0.12. On the 1st day after the end of 'Bilakt' feeding, phagocytic activity increased by 40.9%, the phagocytic index was 3.24 ± 0.1, which is 32.1% higher than the initial level.

Prospects for further research. It is promising to conduct further tests on the drug 'Bilakt' due to its high physiological activity. Additionally, its use in beekeeping during the spring growth and development of bee families shows potential in preventing bacterial and fungal diseases among bees.

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Part 2. Biotechnology

UDC 619:602.3:579.864.1:579.873.13:579.862.1:546.284-31 DOI 10.36016/JVMBBS-2022-8-3-4-3

THE EFFECT OF AEROSIL A-300 ON THE GROWTH OF BACTERIA *LACTOBACILLUS PLANTARUM*, *BIFIDOBACTERIUM ADOLESCENTIS* AND *STREPTOCOCCUS LACTIS*

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Summary. The article presents data on the effect of Aerosil A-300 on the growth of bacteria *Lactobacillus plantarum* No. 7-317, *Bifidobacterium adolescentis* No. 17-316, *Streptococcus lactis* No. 5. The results of studies have shown that the most optimal for the growth of bacteria content of Aerosil A-300 in the environment is 2.0%. It has been found that when Aerosil was added to nutrient media, bacteria grew and actively accumulated a significant number of viable cells: 3.8–4.5×10^7 CFU/cm^3 (control 1.1–2.5×10^7 CFU/cm^3) according to the average technological parameters pH 7.0 and the temperature of 37 °C. Studies have shown that the relative increase in the number of cells, by which we assessed the effect of Aerosil on growth, was in *Lactobacillus plantarum* No. 7-317 (87.5 ± 12.0), which is 23% higher than control, in *Bifidobacterium adolescentis* No. 17-316 — (79.2 ± 11.9), which exceeded the control data by 14%

Keywords: fumed silica, nutrient medium, probiotic strains, cultivation

Introduction. Analysis of the technology of production of bacterial drugs showed that an important and promising direction is to improve the initial — basic stages of production, namely the creation of the most productive conditions for biomass accumulation — optimization of nutrient medium (Gujvinska and Paliy, 2018a, 2018b, Raskoshnaya et al., 2016).

Standardization of production media in terms of nutritional value is one of the main factors determining the quality of the finished product. According to the literature (Gujvinska et al., 2018; Kovalenko et al., 2010; Kozlovska et al., 2012), complex nutrient media are regulated for the cultivation of probiotic strains: hydrolysates and extracts of milk, casein, liver, baker's yeast and muscle. The results of the literature data indicate that these media contain a complete set of 19 amino acids (AMA). According to the literature (Danylenko et al., 2013; Kigel et al., 2003; Poltavska, 2006; Ravliuk and Dekhtiarenko; 2016), the main components of the nutrient medium for growing bifidobacteria are sources of amino acid nitrogen, trace elements (sulfur), vitamins, growth factors, peptones, sodium chloride, components to increase the density of the medium and others.

Yeast is a source of amino acids, growth factors, vitamins; the source of peptones and minerals is casein hydrolyzate; the source of sulfur — sulfur-containing amino acid — L-cystine; because bifidobacteria are strict anaerobes, to increase the density of the nutrient medium, in order to complicate the diffusion of air into the medium, agar is used; sodium chloride is used to maintain the appropriate osmotic pressure of the nutrient medium; Lactose, which is metabolized by bifidobacteria and lactobacilli, is used as a source of sugars.

Lactic acid bacteria are very common in nature (Poltavska, 2006; Ravliuk and Dekhtiarenko, 2016). The study of the biological properties of lactobacilli and bifidobacteria, as well as other microorganisms, requires the ability to long-term preservation of cultures (Kovalenko, 2014; Timchenko, 2010; Yankovs'kiy, 2005). This is necessary both to maintain the collections of lactic acid bacteria in a highly active state, and for the manufacture and storage of probiotics (Kovalenko, 2002; Khyzhniak, 2014).

These circumstances required the search for a new composition of the nutrient medium based on the components from domestic raw materials, which would reduce its cost and increase growth properties, in particular to accelerate the growth of lactobacilli, bifidobacteria and the accumulation of their bacterial mass.

One of the substances that has good adsorbing and stabilizing properties is Aerosil — amorphous anhydrous silicon dioxide, belongs to the group of synthetic active highly dispersed mineral fillers. In pharmacy and bioindustry it is used as an excipient, stabilizer, gelling agent, adsorbent, adjuvant and a substance that improves the fluidity of tablets, ointments, gels and other mixtures.

The aim of our research was to study the effect of Aerosil A-300 in the composition of growth substrates on the growth of bacteria *Lactobacillus plantarum* No. 7-317, *Bifidobacterium adolescentis* No. 17-316, *Streptococcus lactis* No. 5 in their cultivation.

Materials and methods. The study was performed using strains *L. plantarum* No. 7-317, *B. adolescentis* No. 17-316, *St. lactis* No. 5.

Perfection the technology of cultivation of production strains *L. plantarum* No. 7-317, *B. adolescentis* No. 17-316, *St. lactis* No. 5 on different growth substrates was performed with the addition of Aerosil A-300. For this purpose, samples of media for the cultivation of lactic acid bacteria were prepared. Aerosil A-300 was added to them in final concentrations of 1.0%, 2.0%, and 3.0%. After that, lactic acid bacteria at a concentration of 10^7 CFU/cm^3 were added to the medium with Aerosil and cultured at a temperature of 37 °C.

Aerosil 300 (fumed silica, CAS number 112945-52-5) white powder, amorphous, non-porous. Insoluble in water, acids and dilute alkalis. It has good sorption properties. Specific surface area 200 ± 25 m^2/g; the average particle size is 12 μm; the pH of 4% aqueous dispersion is from 3.6 to 4.3. Numerous studies have confirmed that Aerosil has therapeutic effect in diseases of the gastrointestinal tract.

The effect of Aerosil on the growth activity of lactic acid cultures was assessed using the indicator of relative growth of biomass of microorganisms during the daily period of their growth (X):

$$X = \frac{N_{24}}{N_0},$$

where: N_0 — the initial concentration of the cells of microorganisms; N_{24} — the final concentration of the cells of microorganisms.

and the stimulation indicator — relative growth of bacteria in experiment and control (SI):

$$SI = \frac{X_{exp}}{X_{control}},$$

where: SI in the control medium was taken as a unit; X_{exp} — experimental medium; $X_{control}$ — control medium without Aerosil.

The activity of the strains was evaluated by the results of skim milk fermentation for 72 hours and the ability to form acid according to the method of Bannikova (1987).

The number of living microbial cells was determined by serial dilutions of the resulting suspension in saline, followed by seeding of bacterial cultures per 0.1 cm^3 from dilution 10^6 to MRS-4 medium.

All studies were performed in triplicate. Statistical processing of the results was performed according to traditional methods of variation statistics using the program MS Excel and Statistica 10.

Results. It should be noted that modern biotechnology for the production of complex probiotics is based on the separate cultivation of different strains and their subsequent combination in certain proportions.

Given the symbiosis of beneficial bacterial flora in the human body, the peculiarities of the growth of cultures used (oxygen bonding, vitamin production, the need for certain nutrients, etc.), the need for nutrients in the recovery phase in the enteric environment, it is important and expedient to combine deep cultivation of different strains and the introduction of a prebiotic component, which will have a significant clinical effect. In addition, not only living cells are useful, but also the products of their metabolism (organic acids, bacteriocins, vitamins, etc.), which also have a positive effect on biochemical reactions in the body, confirming the need to preserve the culture medium in the combined preparation before drying.

We started the system of maintaining the biological properties of production strains of lactic acid bacteria at a high level and improving the system of their preservation by improving the medium for their cultivation.

The work was carried out to improve the technology of cultivation of production strains of *L. plantarum* No. 7-317, *B. adolescentis* No. 17-316, *St. lactis* No. 5 on growth substrates with the addition of Aerosil A-300, which can significantly stimulate growth activity and production of biologically active substances of some species of bacteria.

During the experiment, samples of media for the cultivation of lactobacilli and bifidobacteria were prepared, to which Aerosil A-300 was added in various concentrations from 1.0% to 3.0%. Then lactobacilli and bifidobacteria were added to the medium with Aerosil at a concentration of 10^7 CFU/cm^3. The cultures were incubated at a temperature of 37 ± 0.5 °C (Table 1).

Table 1 — Study of the growth of bacteria *B. adolescentis* No. 17-316 and *L. plantarum* No. 7–317 in the medium with Aerosil (M ± m)

Bacteria	Indicators	The amount of Aerosil A-300 in the test medium,%			
		1.0	2.0	3.0	Control
L. plantarum No. 7–317	SI	0.36 ± 0.04	0.76 ± 0.15	0.26 ± 0.16	1.0
	pH	6.7	7.1	6.8	7.2
B. adolescentis No. 17-316	SI	0.39 ± 0.08	0.91 ± 0.72	0.26 ± 0.12	1.0
	pH	7.1	6.9	7.2	7.2

Note: the stimulation index (SI) is the relative increase in the number of bacteria in the experiment and control.

The results of studies have shown that the most optimal for the growth of bacteria content of Aerosil A-300 in the medium is 2.0%. Thus, the stimulation index was 0.76 ± 0.15 for lactobacilli and 0.91 ± 0.72 for bifidobacteria.

The next stage of work was the preparation of experimental medium with Aerosil A-300 (2%) for the cultivation of lactobacilli and bifidobacteria.

Studies have shown that the relative increase in the number of cells, by which we assessed the effect of Aerosil on growth, was 87.5 ± 12 in *L. plantarum* No. 7-317, which is 23% higher than control; and 79.2 ± 11.9 in *B. adolescentis* No. 17-316, which exceeded the control data by 14% (Table 2).

Thus, if we take the index of stimulation of bacterial growth in the medium without Aerosil A-300 per unit, then at the optimal concentration of Aerosil administered into the medium (2.0%), it was maximum and amounted to 0.76 ± 0.15 for *L. plantarum* No. 7-317 and 0.91 ± 0.72 — for *B. adolescentis* No. 17-316.

It is known that when lyophilized probiotics are introduced into the intestine, only 10% of bacterial cells attach to its surface, colonizing the mucous membrane, and the rest of the cells are excreted. Therefore, one of the main tasks that arises in the development of effective probiotics is to achieve the maximum number of living cells in 1 dose of the drug. There is a direct relationship between the number of microbial cells of lactic acid bacteria that enter the body and the degree of their adhesion to the intestinal mucosa. The results obtained by us allowed us to conclude about the biocompatibility of probiotic strains of lactic acid bacteria and Aerosil A-300. Therefore, this sorbent is promising for the creation of effective complex drugs. In the combined drug, which will include both a probiotic and a sorbent, a significant proportion of bacterial cells can be adsorbed on the sorbent, forming a larger area of contact with the mucous membrane, and thus enhance the colonization resistance of the latter.

Table 2 — The effect of Aerosil A-300 on the growth of bacteria *L. plantarum* No. 7-317 and *B. adolescentis* No. 17-316 (M ± m)

Medium	Number of microbial cells, $\times 10^7$ CFU/cm^3		Relative cell growth		pH of the medium	
	L. plantarum No. 7-317	*B. adolescentis No. 17-316*	*L. plantarum No. 7-317*	*B. adolescentis No. 17-316*	*L. plantarum No. 7-317*	*B. adolescentis No. 17-316*
Experimental	4.2 ± 0.29	4.5 ± 0.17	87.5 ± 12.0	79.2 ± 11.9	7.1 ± 0.3	6.9 ± 0.2
Control	1.1 ± 0.56	2.5 ± 0.29	67.3 ± 13.0	68.2 ± 12.0	7.3 ± 0.4	7.1 ± 0.3

Note: relative cell growth — initial and final (after 24 h of growth) concentration of cells of the microorganism.

The next step was to study how Aerosil A-300 affects the growth and activity of lactobacilli and bifidobacteria. Studies have shown that the number of microbial cells grown on media with Aerosil increased — *L. plantarum* No. 7-317 ($3.8 \pm 0.29 \times 10^7$ CFU/cm^3) compared to control ($1.1 \pm 0.56 \times 10^7$ CFU/cm^3), *B. adolescentis* No. 17-316 ($4.5 \pm 0.17 \times 10^7$ CFU/cm^3) compared to control ($1.3 \pm 0.46 \times 10^7$ CFU/cm^3), *St. lactis* No. 5 ($3.8 \pm 0.37 \times 10^7$ CFU/cm^3) compared to control ($2.5 \pm 0.29 \times 10^7$ CFU/cm^3), and correlated with the rate of acid production (Table 3). It was found that the rate of acid formation in *L. plantarum* No. 7-317 was 145 ± 20 °T, and in the control group this figure was 115 ± 17 °T. It should also be noted that the rate of acid formation in *B. adolescentis* No. 17-316 was higher on the experimental medium and was 153 ± 30 °T, while in the control — 105 ± 20 °T. Good acid formation was on the experimental medium in *St. lactis* No. 5 — 141 ± 21 °T, and in the control this figure was much lower — 104 ± 17 °T. During the experiment, it was found that the most optimal concentration of Aerosil in the medium is 2.0%. Thus, during 24 hours of growth, the number of lactic acid and bifidobacteria increased and ranged from 3.8×10^7 CFU/cm^3 to 4.5×10^7 CFU/cm^3.

Table 3 — Indicators of activity of lactobacilli and bifidobacteria grown on medium with Aerosil A-300 (M ± m)

Medium	Number of microbial cells, $\times 10^7$ CFU/cm^3			Acid formation, °T		
	L. plantarum No. 7-317	*B. adolescentis No. 17-316*	*St. lactis No. 5*	*L. plantarum No. 7-317*	*B. adolescentis No. 17-316*	*St. lactis No. 5*
Experimental	4.2 ± 0.29	4.5 ± 0.17	3.8 ± 0.37	145 ± 20	153 ± 30	141 ± 21
Control	1.1 ± 0.56	1.3 ± 0.46	2.5 ± 0.29	115 ± 17	105 ± 20	104 ± 17

Conclusions. The possibility of culturing bacteria *Lactobacillus plantarum* No. 7-317, *Bifidobacterium adolescentis* No. 17-316, *Streptococcus lactis* No. 5 on the proposed nutrient medium with the addition of Aerosil A-300 was established.

It was found that the most optimal concentration of Aerosil in the medium for bacterial growth is 2.0%. Growth rates are different: the average activity of acid formation 146 ± 10 °T, the average number of live bacteria — $4.16 \pm 0.27 \times 10^7$ CFU/cm^3; cell morphology is characteristic.

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UDC 619:616.98:578.891:578.2'21:636.4:599.731.11 DOI 10.36016/JVMBBS-2022-8-3-4-4

SEARCH FOR THE PUTATIVE RNA THERMOMETERS IN THE GENOME OF HEPATITIS E VIRUS

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Summary. Currently, some temperature-sensitive elements in bacteria are known. Structurally and functionally different RNA thermometers control a variety of cellular processes in bacteria including virulence. Up-to-date experimental confirmation of RNA thermometers functioning in viruses was obtained only for West Nile virus. But other, unknown yet, types of RNA thermometers may exist in nature. The goal of this study was the determination of conservative stem-loop structures in the swine, wild boars' hepatitis E virus (HEV) genome which may act as RNA thermometers. The search for putative RNA thermometers in the swine HEV which is a common pathogen in the pig population worldwide was executed. Bioinformatics analysis was used to predict the secondary structure of the linear RNA fragments and to determine the melting temperature of the potential hairpins in the HEV genome. 108 swine, wild boars genotype 3 and genotype 4 HEV isolates with complete genomes from the GenBank database were analyzed for the availability of stem-loop structures. Conservative hairpin with the putative thermoregulating function was found in genotype 3 HEV isolates from pig and wild boar for 64 HEV isolates from 108 analyzed ones. The stem of the hairpin with a length of 37 nt contains two AUG start codons of translation initiation and the melting temperature of the hairpin is equal to 38–42 °C for ionic strength of 0.165 M Na+. These hairpins contain a metastable element (one or two bulges) in the stem. Conservative secondary stem-loop structures with putative thermoregulating function for genomic RNA of 64 HEV isolates with complete genome were found by bioinformatics analysis. These hairpins contain a metastable element (one or two bulges) in the stem like an RNA thermometer of West Nile virus and satisfy the necessary and sufficient conditions of RNA thermometer formation. Determined stem-loop structures are proposed as putative thermoregulator elements because they are highly conservative uncanonical structures that are present in the genomes of 64 HEV isolates from 108 analyzed ones

Keywords: hairpin, stem-loop structure, pigs, wild boars

Introduction. Initiation of translation (protein synthesis) is one of the fundamental processes that is regulated through gene expression. Interaction between ribosome and mRNA sequence (Shine–Dalgarno sequence, SD sequence) is required for translation initiation in bacteria. As a rule, AUG start codon of translation initiation is located not further than 15 nucleotides from the SD sequence.

Pathogenic microorganisms often react to temperature increases by induction of expression of virulence genes. At low temperatures, the SD sequence is located within the stem-loop structure formed by the genomic RNA of a pathogen. An increase in temperature destabilizes the stem-loop structure so that the ribosome binding site (SD sequence) becomes available and the translation may be activated.

Currently, some temperature-sensitive elements in bacteria are known. Structurally and functionally different RNA thermometers control a variety of cellular processes in bacteria including virulence.

All the known RNA thermometers are structures with one or two extended stem-loop structure or several ones. Hairpins may be perfect or unperfect (i.e. hairpins may contain mismatched nucleotides in the stem of hairpin). Hairpin with mismatched nucleotides in the stem is shown on Fig. 1.

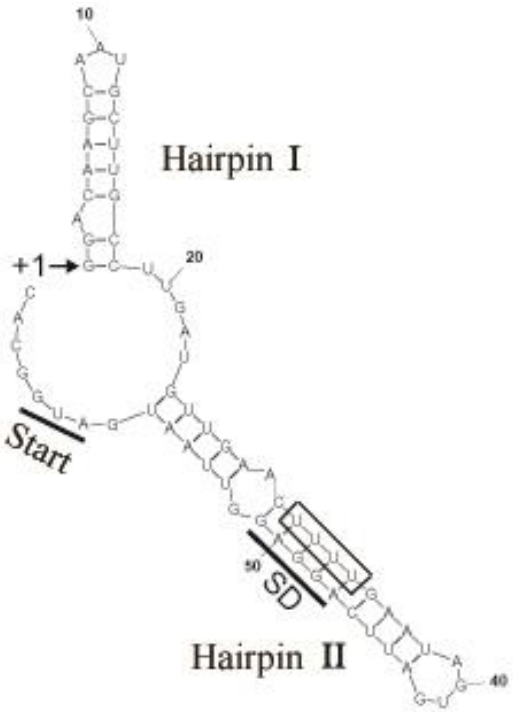

Figure 1. The secondary structure containing the predicted and confirmed 4U RNA thermometer in the *agsA* gene of *S. enterica* (Waldminghaus et al., 2007) at 20 °C. The Shine–Dalgarno sequence in the stem of the hairpin and AUG translation initiation site are highlighted.

Based on bioinformatics analysis of 25 *Salmonella enterica* isolates with complete genomes, the algorithm and criteria for the search for new putative RNA thermometers in the genome of bacteria were developed by the author and coauthors in the paper (Limanskaya et al., 2013).

For *S. enterica* in addition to the known 4U RNA thermometer (Fig. 1) four stem-loop structures were proposed as putative thermoregulator elements (Limanskaya et al., 2013). These hairpins were located in 5'-UTR of virulence regulators *gltB* and *yaeQ*. Predicted elements (SeT1, SeT2, SeT3 (which can be in two different conformations) and SeT4) locate in 5'-UTR, contain SD sequence at a specific distance to translational start codon and their secondary structures are similar to U6 synthetic thermometer structure (Fig. 2).

Experimental checking of four putative thermoregulators was performed in *E. coli* which contained pBSUx plasmid with cloned 5'-UTR RNA thermometers. GFP was used as the reporter gene and the putative thermometer sequence was applied as 5'-UTR. The regulatory role of these 5'-UTR constructs at the translational level was proved by Nothern blot analysis (Fig. 3).

The analysis of RNA and protein accumulation under different temperatures was performed. All tested constructs have shown thermoregulatory function.

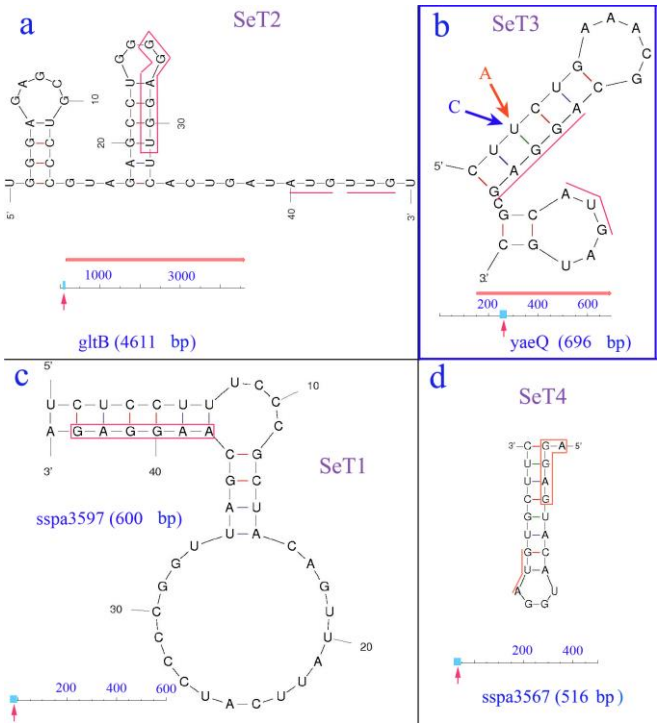

Figure 2. Putative thermoregulator elements in the genome of *S. enterica* are located in 5'-UTR of *gltB* (a — SeT2), *yaeQ* (b — SeT3), *sspa3597* (c — SeT1), *sspa3567* (d — SeT4) genes containing Shine–Dalgarno sequence (in stem-loop structure) and AUG start codon (Limanskaya et al., 2013).

They inhibit GFP expression at 22 °C but allow GFP expression at higher temperatures. All thermoregulators allow GFP expression at 37 °C. GFP expression is strongly induced at 42 °C (fever-related temperature) for all the *Salmonella* thermoregulators. SeT3a and SeT3b have shown clear induction of protein accumulation, but no a significant change in mRNA accumulation (Neupert et al., 2013). Up-to-date experimental confirmation of RNA thermometers functioning in viruses was obtained only for West Nile virus (WNV) (Meyer et al., 2020). But other, unknown yet, types of RNA thermometers may exist in nature.

The goal of this study was the determination of conservative stem-loop structures in the swine, wild boars' hepatitis E virus (HEV) genome which may act as RNA thermometers. The search for putative RNA thermometers in the swine HEV which is a common pathogen in the pig population worldwide was executed.

Materials and methods. The Mfold software package (www.unifold.org) (Zuker, 2003) and Blast (http://blast.ncbi.nlm.nih.gov) were used to predict the secondary structure of the linear RNA fragments and to determine the melting temperature (T_m) of the potential hairpins at the physiological ionic strength (I = 0.2 M Na^+, $[Mg]^{2+}$ = 0.0 mM or I = 0.15 M Na^+, $[Mg]^{2+}$ = 0.2 mM).

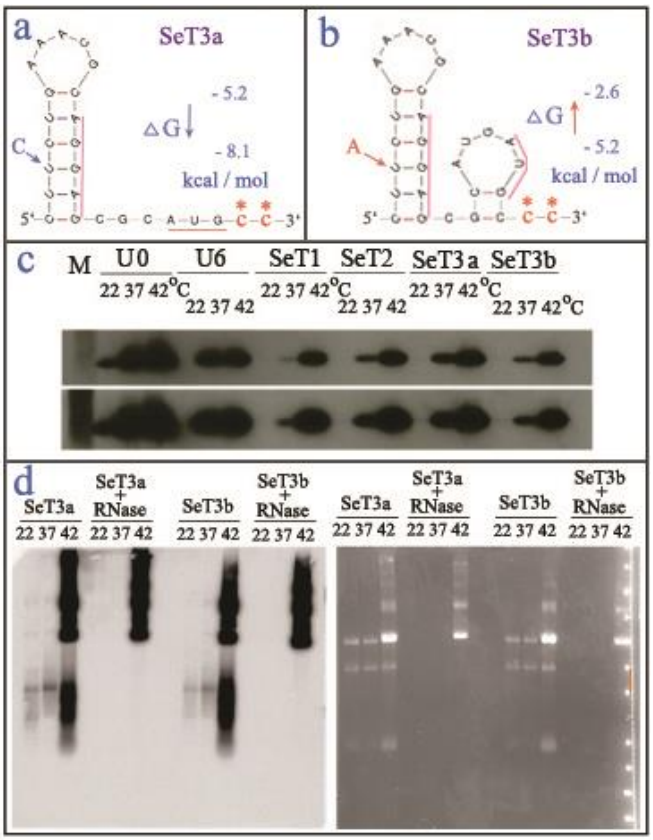

Figure 3. Putative thermoregulator elements from *S. enterica* (a, b) and results of their testing in *E. coli* by Western blot (c) and Nothern blot (d) at different temperatures (Neupert et al., 2013).

108 swine, wild boars genotype 3 and genotype 4 HEV isolates with complete genome from the GenBank database (https://www.ncbi.nlm.nih.gov/genbank) were analyzed for the availability of stem-loop structures.

Previous computer and thermodynamic analyses of RNA thermometers in the *S. enterica* isolates have allowed us to determine the necessary criteria for a potential RNA thermometer. The search for HEV putative thermoregulators was performed for hairpins which are located in 5'-UTR (untranslated region), the melting temperature of the hairpin is within the 37–43 °C range (at the physiological ionic strength), AUG start codon is located in the stem of stem-loop structure.

Results and discussion. Conservative hairpins with putative thermoregulating function were found in genotype 3 HEV isolates from wild boar (Fig. 4a) and pig (Fig. 4b) for 64 HEV isolates from 108 analyzed ones. The stem of the hairpin with length of 37 nt contains two AUG starts codons of translation initiation and its melting temperature is 38–42 °C for ionic strength of 0.165 M Na+. These hairpins contain one (bulge; Fig. 4b) or two (Fig. 4a) metastable elements (buldges; Fig. 4b) in the stem.

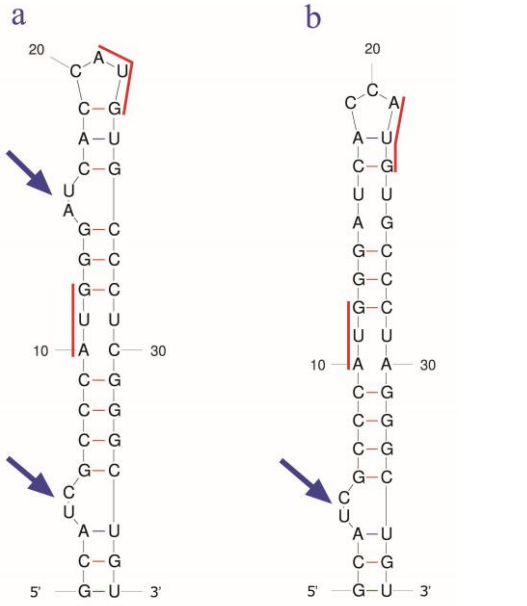

Figure 4. Putative RNA thermometer in the genome of hepatitis E virus with a length of 37 nt. Translation initiation sites AUG are highlighted. Sites AUG are located in the stem and loop of hairpin for genotype 3e HEV isolate from wild boar (a, accession number in the GenBank AB780450, position 5,135–5,171 nt) and genotype 3e HEV isolate from a pig (b, accession number in GenBank MH184579, position 5,156–5,192 nt). Arrows indicate bulges.

Conservative fragment of HEV genomic RNA with a length of 91 nt contains three AUG start codons of translation products (ORF3, ORF2S, ORF2C) of ORF3 and ORF2 viral genes (Fig. 5).

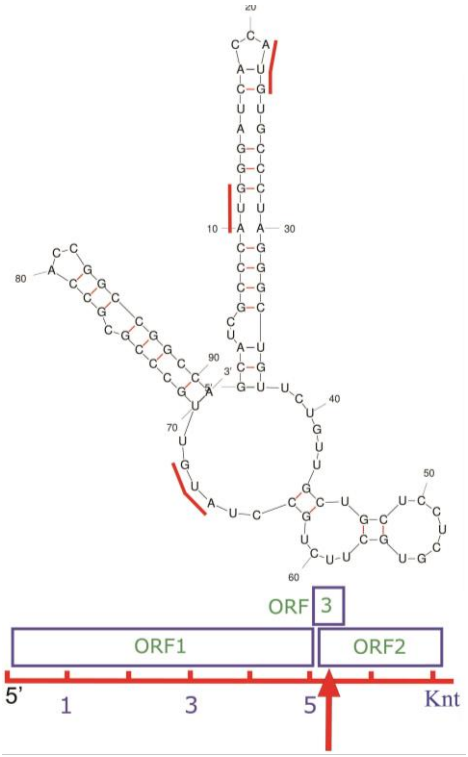

Figure 5. Secondary structure of 91 nt swine HEV fragment with two stem-loop structures including RNA thermometer. Location of putative RNA thermometer on swine HEV isolate F19 with complete genome with length of 7,239 nucleotides (nt; accession number in GenBank MN614429, position 5,147–5,237 nt) is shown by arrows. The viral genome contains three open reading frames (ORFs): ORF1 (position 6–5,132), ORF2 (position 5,167–5,497), ORF3 (position 5,129–5,497).

Determined HEV hairpin is similar to a viral RNA thermometer in WNV (Meyer et al., 2020). The calculated Tm of the hairpin (with a length of 59 nt and stem of 15 bp) of WNV RNA thermometer is equal to 54 °C for 0.2 M Na+ ionic strength. WNV RNA thermometer contains a metastable element, which consists of two conserved base pairs that are flanked by two symmetrical bulges. At the normal temperature of the human body, this RNA thermometer exists in stable conditions that lead to low kinetics of virus replication.

At the favored temperature, AUF1 p45 host protein interacts with the hairpin of WNV and destabilizes stem-loop structure with the following switching from the linear to the circular conformation of the viral RNA and fast pathogen replication. Both for human body and for pig, wild boar the temperature of 42 °C is fatal. We hope that HEV hairpin could interact in the swine body with host destabilizing protein in a similar manner. The authors of the paper (Meyer et al., 2020) explained that their hairpin works as an RNA thermometer that modulates flavivirus replication during host switching.

The Putative HEV RNA thermometer is located in the 5'end of ORF2 gene (ORF, open reading frame). The

ORF2 is located at the 3' end of the genome and encodes the major capsid protein that contains immunogenic epitopes, induces neutralizing antibodies, and is the target for vaccine development (Jameel et al., 1996; Xing et al., 2011). ORF2 contains an endoplasmic reticulum signal peptide (Graff et al., 2008). Three glycosylation regions have been identified in ORF2, but the biological relevance of these potential modifications is unclear (Pérez-Gracia et al., 2015).

The ORF2 capsid protein interacts with the 5' end of the viral RNA which probably plays a role in the viral RNA encapsidation (Surjit et al., 2004). Recent reports suggested that ORF2 protein is processed into at least two forms, including one or two forms of secreted glycoproteins that are not associated with infectious particles, and one unglycosylated form which is the structural component of infectious particles (Yin et al., 2018; Montpellier et al., 2018).

All ORFs are expressed during viral infection, as antibodies against these regions have been detected in naturally infected humans and experimentally infected monkeys (Khudyakov et al., 1994). Testing putative HEV RNA thermometer may be performed as in the paper (Meyer et al., 2020). Differential melting curves for HEV hairpins to determine melting temperature as of whole hairpin as of bulges at physiological ionic conditions may be performed for preliminary checking. To obtain more functional data, experiments with some mutants that stabilize or destabilize the thermometers may be performed.

Conclusions. Conservative secondary stem-loop structures with putative thermoregulating function for genomic RNA of 64 HEV isolates with complete genome were found by bioinformatics analysis. These hairpins contain a metastable element (one or two bulges) in the stem like an RNA thermometer of West Nile virus and they satisfy the necessary and sufficient conditions of RNA thermometer formation.

Importantly, the determined stem-loop structures (Figs 4–5) are highly conservative uncanonical structures that are present in the genomes of 64 HEV isolates from 108 analyzed ones. They may control translation initiation in the eukaryotic host like a similar RNA thermometer of WNV controls virus replication in the host.

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Part 3. Biosafety

UDC 619:604.6:633.1/.3:636.085.9:577.2.08 DOI 10.36016/JVMBBS-2022-8-3-4-5

OVERVIEW OF THE ISSUE OF GENETICALLY MODIFIED CROPS IN UKRAINE

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Summary. The issue of regulating the circulation of genetically modified (GM) crops and their products is extremely important for Ukraine. This is confirmed by climate change, which indicates the need for rapid adaptation of existing varieties while maintaining the yield level; increasing pest resistance to pesticides; international competitiveness of GM products; the need to comply with regulations on genetically modified organisms (GMOs) for European integration and the presence of genetically modified seeds in the country's crops. So, the purpose of the work was to consider the problems, prospects and potential of GM plants in Ukraine. Qualitative analytical methods were used in the market analysis. Information was obtained from official data sources and market surveys. The results of four-year screenings in Dnipropetrovsk Region were also summarized. PCR diagnostics was used as verification method. In the course of the work, it has been established the presence on the Ukrainian market of more than two dozen GM soybean varieties, four transgenic sunflower hybrids, and ten transgenic corn hybrids from the world's leading producers of Canada (Bramhill seeds, Sertis Holding S.A., Hyland Seeds, Sevita Int., Prograin), the USA (Asgrow & Monsanto), France (R.A.G.T.), Austria (Saatbau Linz). During 2018–2021, the distribution of transgenic products among domestic products in Dnipropetrovsk Region has been recorded. Thus, real-time PCR revealed that GMOs were present in 42.8% of the analyzed soybean samples; 87.5% of mixed fodder; 15.0% of sunflower samples. It has been established that the circulation of falsified GM products on the country's market ranged from 25 to 50% (inconsistency in marking, certificate, holograms, and QR code), which indicates the imperfection of legal regulation and creates prerequisites for their illegal use

Keywords: soybean, corn, sunflower

Introduction. The law 'On the state biosafety system for the creation, testing, transportation and use of genetically modified organisms' was adopted in 2007, but discussions on the issue of legal circulation of GMOs in Ukraine continue (Slasten, 2020). For example, there are no registered genetically modified varieties or hybrids in the country today. And this is at a time when the share of GM cultures in the crops of farmers in the USA, Canada, Brazil, Argentina, and Australia already reaches more than 90%, and most of the EU countries import GM corn and soybeans for animal husbandry without any problems.

Taking into account such world trends, the issue of regulating the circulation of GM crops and their products is extremely important for Ukraine. This is confirmed by climate change, which indicates the need for rapid adaptation of existing varieties while maintaining the yield level; increasing pest resistance to pesticides; international competitiveness of GM products; the need to comply with regulations on GMOs for European integration and the presence of genetically modified seeds in the country's crops.

Therefore, Ukraine, as one of the largest suppliers of agro-food products to the world market (FAO …, 2022), needs to adapt to these changes now. The world's concern about changes in weather and climate conditions is pushing countries to implement important transformations, including in agriculture. National programs such as the Green Deal are being developed and implemented to help slow global warming by reducing greenhouse gas emissions, deforestation and other measures.

Measures taken by international organizations already affect the conditions of agricultural activity and trade in agricultural products. One of the trends in the European Union is Carbon footprint — carbon policy and everything that happens around it. Soybean and its processing products play an important role in this issue, since 22% of CO_2 emissions in the EU agricultural production are due to this crop (Shevchenko, 2021).

The recognition in 2020 (Nurton, 2020) by the Nobel Committee of Scientists for the development of a method of genome editing using CRISPR/Cas9 is an important step on the way to the cultivation of new varieties of agricultural crops that are already entering national commercialization regulatory schemes. Such changes were implemented for the first time in Argentina, which indicates significant shifts in the field of agricultural biotechnology products. Changes to the law on gene-edited crops show that laws must adapt to changing conditions (Turnbull, Lillemo and Hvoslef-Eide, 2021).

European scientists are also calling for changes to European legislation on GMOs, which they say is necessary for the further development of sustainable agriculture, ensuring sufficient food for the population and protecting the environment (Šaradínová, 2020). 'Genome editing that leads to changes that can also occur spontaneously in nature and that do not introduce foreign DNA should, in addition, be exempted from the application of GMO legislation', said a statement by EU-SAGE, which brings together members from 132 European research institutes and associations.

185.1 million hectares in 26 countries (as of 2016) are planted with GM crops. Undoubtedly, the world leader in the cultivation of GM crops is the USA. Currently, ten crops are commercially grown there: zucchini (since 1995), soybeans (since 1995), corn (since 1996), cotton (since 1996), papaya (since 1997), rapeseed (since 1999), alfalfa (since 2006), sugar beet (since 2006), potatoes (since 2016), apple (since 2017). The top five countries (as of 2017) also include Brazil, Argentina, Canada and India. Other GM crops currently approved for commercial use in various countries are eggplant, sweet pepper, carnation, petunia, rose, tobacco, tomato, wheat, sugar cane, bean, chicory, mung bean, agrostis, eucalyptus, flax, melon, plum, turnip, rice, safflower, poplar. At the same time, 1–3 genetic modifications have been registered for some of them (for example, petunias, roses), and for some — tens or even hundreds (for example, soybean — 42, cotton — 63, corn — 231). In terms of volume, GM soybeans are grown the most in the world, followed by corn, and third by cotton (Useful Information, no data).

The development of the Ukrainian soybean market is significantly influenced by the trends of the main buyer countries, since Ukraine is a net exporter, consuming only up to 20% of the produced volumes (Shevchenko, 2021). The key buyers of these products in the world are China — with a consumption volume of 100 million tons per year and the EU — about 40 million tons. China plans to accelerate the introduction of genetically modified corn varieties, which could allow Chinese farmers to start planting GM corn as early as 2023 (China …, 2022).

The experience of other countries shows that it is advisable to regulate the possible impact when using modified organisms in accordance with the 'Cartagena Protocol' (SCBD, 2000). Thus, Ukraine joined the Cartagena Protocol on Biosafety to the Convention on Biological Diversity on September 12, 2002 (VRU, 2002).

The spread of GM plants has become an irreversible process (Rudyshin, 2009). Thus, the Agent Green association (Romania) with the assistance of the 'Danube Soya' association conducted field research in 2018. 60 large fields in the six main soybean regions of Ukraine were investigated. The share of GM soybeans was 48%. Despite the ban, GM crops are actively grown in Ukraine. There is absolutely no control over the cultivation of GM soybeans, although the products obtained using this technology are, according to the law, subject to complete destruction (There is …, 2017).

So, the work aimed to consider the problems, prospects and potential of GM plants in Ukraine.

Materials and methods. Qualitative analytical methods were used in the market analysis. Information was obtained from official data sources and market surveys.

Laboratory analyzes were carried out by an accredited laboratory (ISO 17025) in Dnipro, based on officially approved methods of analysis. Samples were tested to determine compliance with food safety parameters, to provide early warning of potential problems, and to provide a basis for risk assessment.

Real-time PCR laboratory screening covered raw materials, semi-finished products and finished products for export and domestic production (n = 410). 35 S promoter of cauliflower mosaic virus, NOS terminator of *Agrobacterium tumafaciens*, and FMV 34 S promoter were determined in the samples.

Test results were documented and subjected to regular review and trending. The test samples were selected for the purpose of official control in compliance with the established rules and standards of selection and analysis. Samples were handled and labeled in a manner to ensure legal and analytical validity. The results were interpreted according to the criteria defined by the legislation.

Results. Taking into account the focus of Ukrainian farmers on soybeans and planning to increase its area to 1.4 million hectares (Panasiuk, 2022), an online analysis of transgenic seed material in the country was made. The availability of 24 GM soybean varieties (Table 1) from the world's leading producers from Canada (Bramhill seeds, Sertis Holding S.A., Hyland Seeds, Sevita Int., Prograin), USA (Asgrow & Monsanto), France (R.A.G.T.), Austria (Saatbau Linz) has been established on the market of Ukraine. The turnover of falsified products on the market was 25% (mismatch of marking, certificate, holograms and QR code).

Video reports from Poltava Region testify to the cultivation of GM soybean varieties — Sinara, Histar in 2022 (Volovyk, 2022; SH Zhyttia, 2022), Kansas (Alex_Agro, 2021) in 2021; from Sumy Region — Apollo, Ultra in 2021 (Sumshchyna, 2021); from Chernivtsi Region — Whitby in 2019 (Hrytsyk, 2019); from the city of Zaporizhia — Maximus in 2021 (Selhozperedelkin, 2021); from Crimea — Sigalia in 2021 (Ernest-No-Till, 2021).

The sale of four transgenic sunflower hybrids (Table 2) from two of the world's leading producers from Canada (Sertis & DOW Chemical, Union Carbide & Sertis Holding S. A.) has also been established. The turnover of falsified products on the market was 25%.

Sales implementation of ten transgenic corn hybrids (Table 3) from two of the world's leading producers from Canada (Sertis Holding S.A. & DOW Chemical, Union Carbide & Sertis Holding S.A.) was established. The turnover of falsified products on the market was 50% (mismatch of marking, certificate, holograms and QR code).

Table 1 — GM soybean seed material on the Ukrainian market in 2022

Variety, yield centner/hectare	Manufacturer / country	Source of information	Region of Ukraine
Colby, 45–68	Sertis Holding S. A. / Canada	https://agrodobro.com.ua/goods/semena-soi-colby/	Dnipro
Whitby, 55–60		https://flagma.ua/uk/semena-soi-uitbi-whitby-ultrarannyaya-o12863487.html	Khorol, Poltava Region
		https://agrovektor.com/physical_product/765050-semena-soi-whitby.html	Kharkiv
Histar, 42		https://flagma.ua/uk/semena-soi-haystar-histar-sredneranney-105-o11331291.html	Khorol, Poltava Region
Sydney Bt, 72		https://certisgroup.eu.com/product/soya-sydney-bt-nano-transgennaya https://flagma.ua/novy-kanadskiy-elitny-sort-soi-sydney-bt-o4798252.html	Odesa
Merlin, 30–35	Saatbau Linz / Austria	https://favorit-td.com.ua/p8641250-soya-gmo.html https://agro-liga.com/catalog-produkcii/soya-merlin/	Kropyv-nytskyi
Maximus, 47	Monsanto / USA	https://agrovektor.com/physical_product/83029-semena-soi-maksimus-gmo.html	Poltava Region
Maxus, 50	Asgrow & Monsanto / USA	https://favorit-td.com.ua/p376240947-soya-maxus.html https://agrovektor.com/ua/physical_product/598929-soya-gmo-maksus.html	
Конор, 42	Hyland Seeds / Canada	https://agrovektor.com/physical_product/194138-semena-soi-konor.html	Kharkiv
Madison, 45		https://favorit-td.com.ua/p376160466-soya-madison.html	Pidhaitsi, Kirovohrad Region
AOC Calipso, 58	Sevita Int. / Canada	https://favorit-td.com.ua/p375938205-soya-aoc-calypso.html	
Silesia, 45	Prograin / Canada	https://favorit-td.com.ua/ua/p376011986-soya-silesia.htm	
DH 530, 55	Sevita Int. / Canada	https://favorit-td.com.ua/p375927893-soya-530.html	
DH 618, 40–48		https://favorit-td.com.ua/p375912258-soya-618.html	
Kyoto	Prograin / Canada	https://favorit-td.com.ua/p376045820-soya-kyoto.html	
Kanata		https://favorit-td.com.ua/ua/p376034696-soya-kanata.html	
Sultana, 45	R.A.G.T. / France	https://favorit-td.com.ua/p375955629-soya-sultana.html	
Sigalia, 45		https://favorit-td.com.ua/p375981284-soya-sigalia.html	
Kassidy, 58	Prograin/ Canada	https://favorit-td.com.ua/p376058526-soya-kassidy.html	
AOC Drayton, 58	Bramhill seeds/ Canada	https://favorit-td.com.ua/p375946771-soya-aoc-drayton.html	
Ultra, 40	Asgrow & Monsanto / USA	https://favorit-td.com.ua/p376230696-soya-ultra.html	
Apollo, 38		https://favorit-td.com.ua/p376223889-soya-apollo.html https://agrovektor.com/physical_product/194141-semena-soi-apollo.html	
Sinara, 50	R.A.G.T., France	https://favorit-td.com.ua/ua/p376302280-soya-sinara.html	
Kansas, 75	Sertis Holding S. A. & DOW Chemical / Canada	https://dobrosvt.com.ua/goods/semena-soi-kansas-kanadskoy-selektsii/	Kyiv
Oldham Bt, 55-60		https://academsx.com.ua/oldham-bt-1r/	Kherson

Table 2 — GM sunflower seed material on the Ukrainian market in 2022

Variety, yield centner/hectare	Manufacturer / country	Source of information	Region of Ukraine
VIKING F 696, 47	Sertis Holding S. A. & DOW Chemical / Canada	https://agrofermer.com/products/podsolnechnik/	Odesa
CARRON, 48			
LEBRON, 53			
NUBIRA F 369, 52	Union Carbide & Sertis Holding S. A./ Canada	https://zakupka.com/p/1311529572-podsolnechnik-gmo-gibrid-nubira-f369-konditerskiy/	

Table 3 — GMO corn seed material on the market of Ukraine in 2022

Variety, yield centner/hectare	Manufacturer / country	Source of information	Region of Ukraine
TOPEKA F-36 FAO 250, 180	Sertis Holding S. A. & DOW Chemical / Canada	https://zakupka.com/p/1333373007-kukuruza-gmo-gibrid-topeka-f-36-fao-250/?e=	Odesa
VALDES BT 199 FAO 180, 180	Union Carbide & Sertis Holding S. A. / Canada	https://zakupka.com/uk/p/1306811415-kukuruza-gmo-gibrid-valdes-bt-199-fao-180/	
HIDRA FAO 250, 250		https://dobrosvt.com.ua/goods/semena-kanadskoy-kukuruzy-hidra/	Kyiv
HIDRA FF-369		https://agrovektor.com/physical_product/77850-kanadskiy-transgennyy-gibrid-kukuruzy-hydra-ff-369.html	Kharkiv
CORBIN FS-899, 180		https://dobrosvt.com.ua/goods/semena-kanadskoy-grechihi-korbin/	Kyiv
SKEENA FF-199, 260		https://dobrosvt.com.ua/goods/semena-kanadskoy-kukuruzy-skeena/ https://agrovektor.com/physical_product/1040846-transgennyy-gibrid-kukuruzy-skeena-ff-199.html	Kharkiv
OTMI FS 466, 190	Sertis Holding S. A. & DOW Chemical / Canada	https://agrovektor.com/physical_product/809843-kanadskiy-transgennyy-gibrid-kukuruzy-otmi-fs-466.html	
ADEL FAO 260	Sertis Holding S. A. / Canada	https://agrodobro.com.ua/goods/semena-kukuruzy-adel-fao-260-18-kg/	Dnipro
WEST FAO 180		https://agrodobro.com.ua/goods/semena-kukuruzy-west-fao-180-kanadskiy-transgenniy-gibrid-18kg/	
POINT FAO 330		https://agrodobro.com.ua/goods/semena-kukuruzy-point-fao-330/	

The results of laboratory experiments (n = 410) for 2018–2021 in Dnipropetrovsk Region have been analyzed. During qualitative analysis of GM, positive samples (n = 21) were found exclusively in products of domestic production (Table 4).

Thus, the level of GM soybean in the samples was 42.8% (n = 7), which was evidenced by the detection of the GM soybean DNA target sequence containing the Cauliflower mosaic virus 35 S promoter / *Agrobacterium tumefaciens* NOS terminator. The level of GM sunflower was 15.0% (n = 54), and the level of GM mixed fodder was 87.5% (n = 8), as evidenced by the detection of the target sequence of GM plant DNA containing the Cauliflower mosaic virus 35 S promoter / terminator NOS of *Agrobacterium tumefaciens.*

Table 4 — Research in Dnipropetrovsk Region during 2018–2021

Year	A total of samples were examined, n	Type and number of positive samples, %	Total GM samples, n
2018	8	soybean texturat (50)	1
2019	61	mixed fodder (100), soy protein (100)	3
2020	163	rapeseed cake (100); mixed fodder (75), yeast (67)	7
2021	178	meal (sunflower concentrate) (15); mixed fodder (100); soybean oil (25)	10
	410		21

In order to study the potential of transgenic crops in Ukraine, a comparison of their yield, using the Whitby variety as an example, with the traditional soybean variety was conducted. Thus, we analyzed the data of the 2018 report (Koretskyi, Derzhanivskyi and Shatsman, 2019) on the study of yield at the test ground for practical field studies using the example of the ultra-early soybean variety Favorit. The experiments were conducted in the Yagotyn district of Kyiv Region, a zone of sufficient moisture in the Left Bank Forest Steppe. The type of soil is typical light loamy chernozem. The humus content was 3.15%, which characterized the soil as sufficiently fertile. The weather conditions of 2018 were quite favorable and typical for the area of research on growing crops. When calculating the hydrothermal moisture coefficient for the growing season of 2018, its value was 1.58, which indicated a good supply of moisture and heat.

Table 5 — Comparison of productivity of ultra-early soybean varieties in Ukraine

Productivity indicators	Productivity of ultra-early soybean varieties without fertilizers	
	Favorit*	Whitby**
Yield, centner/hectare	26.3	36.5
Mass of 1,000 grains, g	133.7	134.0

Notes: * — according to the 2019 report of the independent scientific research agrarian platform Field of Knowledge; ** — according to field data of 2019, Chernivtsi Region (Hrytsyk, 2019)

So, the cultivation of the GM soybean variety Whitby made it possible to obtain higher productivity indicators, namely: to obtain an increase in the yield — 10.2 centner/hectare; to increase the yield level by 38.8%.

It was also reported that the Whitby variety had been grown in 2017 in Sumy, Kirovohrad, Dnipropetrovsk, Cherkasy, Kyiv, Chernihiv, Zhytomyr, Odesa regions of Ukraine (Whitby …, 2022) with a yield of 73 centner/hectare. The Oldham variety yield in 2019 in Kirovohrad, Dnipropetrovsk, Kherson, Vinnytsia, and Zhytomyr regions (Canadian …, 2022) was 76 centner/hectare.

Analysis of the country's market also showed the absence of genetically edited products on the market.

Discussion. According to the results of laboratory experiments in 2018–2021, the level of detection of GM soybeans in Dnipropetrovsk Region was 42.8%, which corresponds to the data published on the official website of the US Department of Agriculture, about the cultivation of 50–65% genetically modified soybeans by Ukrainian producers (GMO …, 2021), as well as and with other sources (Growing …, 2020; Shevtsova, Gerilovych and Solodyankin, 2014).

The presence on the market of Ukraine of more than two dozen GM soybean varieties from 8 leading producers in the world from Canada, the USA, France, Austria, testify to the demand — the need of the market for the specified soybean seeds. Thus, for 10 seasons from 2010 to 2020, an increase in the area of soybean crops was recorded in 16 regions of Ukraine. This is evidenced by data from a dynamic infographic of soybean acreage from SuperAgronom.com (Soybean …, 2020).

The three leaders with more than 125,000 hectares of planted areas for growing this crop were: in the forest-steppe zone — Khmelnytskyi and Poltava regions, and in Polissia — Zhytomyr Region. Thus, Ukraine, as one of the largest producers of soybeans in Europe, needs to technologically adapt to reduce CO_2 emissions from this crop. According to Prof. Dolezel, the positive impact of genetically modified crops on the environment cannot be ignored either. In 2018 alone, 23 billion kilograms less carbon dioxide entered the atmosphere thanks to the cultivation of genetically modified crops (Šaradínová, 2020). It is known that there are about four thousand soybean diseases in the world, of which more than 400 occur in Ukraine. Thus, the cultivation of resistant varieties obtained through genetic modification led to a reduction in the consumption of toxic pesticides by 776 million kilograms worldwide between 1996 and 2018 (Šaradínová, 2020).

In 2021, the average soybean yield, according to the Ministry of Agrarian Policy and Food, was 26 centner/hectare. Officially, it was not GM, but the unofficial 'grey' market of GM soybeans reached 60–80% of crops in different years (Korol, 2022).

Therefore, the presence of 24 varieties of GM soybeans on the market of Ukraine and their cultivation; permission of the European Commission in 2019 to use in the EU 8 varieties of grain and oil crops with GMOs for food and feed purposes (The European …, 2019); the report (NASEM, 2016) (on 606 pages) of the USA in 2016 on the safety and better yields of GM crops (Shutova and Panchin, 2021) testify to the expediency and necessity of regulating the cultivation of proven modified varieties in Ukraine.

Thus, according to the results of laboratory studies, the level of detection of GM sunflower meal in Dnipropetrovsk Region was 15%, which indicates the illegal use of unregistered GM products in Ukraine.

The demand for GM corn is evidenced by the presence on the market of Ukraine of appropriate varieties from Union Carbide & Sertis Holding S.A. from Canada, which is confirmed by the data of the US Department of Agriculture — GM corn crops in Ukraine amount to 1%, and according to other data, 3–5% (World …, 2021). Due to their high energy content, corn and soy are indispensable components of mixed fodder, which may explain the high level of GM samples in the region among mixed fodder — 87.5%. Thus, according to

Ya. and M. Pariiv, in the near future, Ukraine will be forced to work with genetically modified corn, due to the increased harmfulness of the corn beetle, which in the next five years risks becoming a serious problem for agricultural producers (Growing …, 2020).

According to Paul McDivitt reports, the yield of GM varieties is 5.6–24.5% higher than conventional varieties of corn. GM crops are also characterized by a lower percentage of mycotoxins (28.8%), fumonisins (30.6%) and trichothecenes (36.5%), which lead to economic losses and harm human and animal health (Smyth, 2018). Data on the circulation of falsified GM products on the country's market are confirmed by other reports (Fermer Kubani, 2019; My Agro Canada, 2021).

Recognition of the safety of genetically edited products in a number of countries (Japan …, 2019; Genetically …, 2021) indicates the need for legislative regulation of the issue of crops with edited genes in Ukraine as well.

Therefore, the introduction of registration of GM sources will ensure awareness for further protection of the health of people, animals and the natural environment, will create conditions for the safe practical use of GMOs for economic purposes, will enable to prevent the uncontrolled use of GM sources in feed (Nasar et al., 2019).

Conclusions. A review of the GM crop market of Ukraine showed the presence of twenty-four GM soybean varieties, four transgenic sunflower hybrids and ten transgenic corn hybrids from eight leading world producers from Canada, the USA, France and Austria, which indicates consumer demand for products.

The spread of transgenic products among domestically produced products in Dnipropetrovsk Region during 2018–2021 was recorded. Thus, real-time PCR revealed that GMOs were present in 42.8% of the studied soybean samples; 87.5% of mixed fodder samples; 15.0% of sunflower samples.

The circulation of falsified GM products on the country's market has also been established in the range of 25–50% (inconsistency in marking, certificate, holograms and QR code), which indicates the imperfection of legal regulation and creates prerequisites for its illegal use.

Acknowledgements. The author is grateful to O. H. Malimon for providing reporting data on the results of laboratory-diagnostic studies for 2018–2021.

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UDC 619:616.98-085:578/579:615.281.038:546.46-31-022.532:636.4 DOI 10.36016/JVMBBS-2022-8-3-4-6

'NANOVIROSAN' ANTIMICROBIAL COMPOSITE, DESIGNED FOR EMERGENCY EPIZOOTIC SITUATIONS AND SAFE USAGE IN ECOLOGICAL PIG FARMING

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Summary. Analytical data of preclinical and clinical trials of the experimental veterinary composite drug 'NanoViroSan' (containing Methisazone, Silgeran and magnesium nanooxide) on laboratory models of Aujeszky's (AD) and Teschen (TD) diseases, circovirus infections (PCV-2) and actinobacillary pleuropneumonia (APP) as well as in enzootic foci of mixed infection of AD-PCV-APP and swine pox, are presented, respectively. At the level of statistical probability $p \leq 0.01–0.03$ ($n = 88$), the absence of cytotoxic ($n = 40$, cultures of pig testicle cells and pig alveolar macrophages) and biotoxic effects ($n = 48$ guinea pigs) was proven, as well as high antimicrobial (viro- and bacteriostatic) activity of the drug in the concentration range (by Methisazone) of 1.0–4.0 mg/cm^3. Intramuscular administration of the drug to male pedigree piglets in doses of 0.5 cm^3/20 kg and 1.5 cm^3/20 kg three times with an interval of a day made it possible to stop the carriage of the causative agents of mixed infection in the conditions of pig-breeding ($n = 26$, $p \leq 0.02$). Similar treatment with the drug in a dose of 2 cm^3/20 kg (by Methisazone) of a boar and five sows in another commodity farm made it possible to break the chain of vertical transmission of the causative agent of swine pox from the nucleus to offspring of the herd ($n = 227$, $p \leq 0.03$). There conclusion was made regarding the perspective of experimental drug for bioprotection of pig farming in the conditions of martial law, as well, if additional research will be positive — as for the development of permaculture ('green technologies') in the field of pig breeding

Keywords: bioprotection, porcine virobacterial infections, swine pox, permaculture

Introduction. Under the current state of the world economy, agricultural export is becoming an increasingly popular and profitable business (The World Bank, 2022; De Zeeuw and Klank, 1997), and the war of the russian federation has highlighted the crucial role that Ukrainian agricultural exports play in ensuring global food security (FAO, 2022).

Quarantine restrictions on cross-border infections — African swine fever (ASF), foot-and-mouth disease (FMD), Aujeszky's disease (AD), etc. (RIRDC, 2008) and in addition microbial and antibiotic contamination of pig carcasses, pork, and reproductive materials are well-known problems in the export of pig products (Monger et al., 2021; APHA, 2022).

In the system of biological safety of industrial pig farms, the most important objects of control are undoubtedly boars' semen, as well as rearing young animals. After all, it is through them not only the genetic material is exchanged between different pig farms, but also infectious agents — sometimes even the ASF virus (Schulze et al., 2015; Maes et al., 2008; Gallardo et al., 2015). In this sense, the weakly virulent variants of these pathogens pose the greatest danger. Breeding boars, due to the most powerful constitutional endurance compared to all other technological groups of pigs, usually, as if not the last of the herd, show clinical signs of the disease (Müller and Brem, 1991; Henryon et al, 2003). Therefore, they often become a source of long-term persistent infection of industrial herds in the form of latent carriers (Maroto Martín et al., 2010; ANSES, 2018). This is very dangerous for agricultural exports: although the pathogen is 'weak', but with further passage in the herd, sooner or later it becomes the trigger of an epizootic outbreak, if it's not neutralized in the nucleus of the herd — in a group of boars-breeders/their ejaculates as well in repair stock, primarily (Monga and Roberts, 1994). Moreover, it is known that the contamination of boar semen by *Escherichia coli* can even lead to auto agglutination of spermatozoa and, as a result, to the infertility of sows (Buzun et al., 2022).

Earlier, we published data on the effectiveness of the first two experimental drugs 'SuiViroSan' for the rehabilitation of breeding boars and their sperm from the status of carriers of pathogenic microflora (Eggers et al., 2021).

The purpose of this article is to analyze the data of our trials of a medicament of this line with more wide antimicrobial range — of the composite veterinary drug (CVD) 'NanoViroSan' with activity against the swine pox virus (SPV), porcine circovirus type 2 (PCV-2), *Actinobacillus pleuropneumonia* (APP) and especially against Aujeszky's disease virus (ADV) and Teschen

disease virus (TDV) which are models of ASF and FMD pathogens, respectively (Tarka and Nitsch-Osuch, 2021; WHO Expert Committee on Specifications for Pharmaceutical Preparations, 2002).

Material and methods. *Test substance and reference drugs.* CVD 'NanoViroSan' (batch: 98/27-01-2021, total size 27,540 doses or 12.7 l) is a composite veterinary medicine (Table 1), which includes manufactured in Ukraine veterinary drugs 'Methisazone' and 'Silgeran', as well as integrative additives with magnesium oxide nanoparticles enhancing their effect (patent pending). The quality of the composite pharmaceuticals was guaranteed by the relevant Ukrainian technical specifications (UkrTS/UkrNDNC) corresponding with requirements of the Good practices for national pharmaceutical control laboratories (SCVMU, 2007).

Table 1 — Characteristics of the ingredients of the 'NanoViroSan-L/O' veterinary drug

No.	Composite components and their functions		
	Composite components	Veterinary drug or substance / their function	Source
1	Methisazone	Methisazone (N-methyl-isatin-β-thiosemicarbazo-ne) TU U/UkrNDNC 20.4-25945042270-001:2021, a yellow powder insoluble in water, has a pronounced antiviral effect in vitro in doses that are 100–1,000 times lower than toxic. It inhibits the reproduction of both DNA- and RNA-containing viruses (adenoviruses, herpesviruses, poxviruses, paramyxoviruses, influenza viruses of types A and B, retroviruses, etc.)/Antiviral active substance	Laboratory of Immunology of the Institute of Animal Biology NAAS (Lviv, Ukraine)
2	'Silgeran-W'	'Silgeran-W' (TU U/UkrNDNC 10.9-2661009934-001:2016), Natural complexes of glycooligopeptides and lipopolysaccharides of bacterial origin, mixed with supramolecular complexes of zinc and selenium, citrate and sodium formiate; destroys biofilms of pathogenic microorganisms and prevents their formation, enhances the effect of antibiotics, prevents the chronicity of infections, has pronounced antiviral properties against RNA-containing viruses/Antibacterial active substance	SPC 'Ariadna' Ltd. (Odesa, Ukraine)
3	Magnesium Oxide (MgO) Nanoparticles	MgO nanoparticles has a pronounced antiviral effect in vitro in doses that are 1,000 times lower than toxic, help to destroy biofilms of pathogenic microorganisms/Enhancer of composite's antimicrobial activity (patent application)	NPU 'KhPI' (Kharkiv, Ukraine)
4	Vaccine oil	Commercial vaccine oil which is emulsified by hand shaking/prolonging substance (patent application)	Kyiv, Ukraine

They however unlikely encompass the full scope of pharmacological active moieties. Numerous clinical studies have proven the efficacy of these composites in treating swine with different viral and bacterial infections. It is recommended in clinical guidelines (reviewed in approved info of manufacturers).

Infection agents and their maintenance. There, the strains of infectious agents that are relevant for Ukraine, isolated from pigs of commodity farms (all pathogens, with the exception of *Teschovirus*) and the domestic sector (*Teschovirus*) were used in the work. Aujeszky's disease virus (ADV; strain 1082, epizootic, isolate in Donetsk Region on 12.03.2011), Teschen disease virus (TDV; strain 'Butcha', epizootic, isolate in Kyiv Region on 21.09.2004), porcine circovirus type 2 (PCV-2, strain 'I-1024'; epizootic, isolate in Kherson Region on 23.04.2010) and *Actinobacillus pleuropneumonia* type 8 (APP; strain 'Okhtirka-1411', epizootic, isolate in Sumy Region on 07.11.2016) were obtained from the Museum of Strains of Microorganisms of the NSC 'IECVM' (National Property of Ukraine, Kharkiv, Ukraine). These microorganisms were identified and validated according to current requirements (SPOU and NASU, 1995), which correspond to international standards (WIPO, 1980). The specified strains are maintained according to the relevant passport data and Standard Operating Procedures of the NSC 'IECVM'. For testing, under appropriate biosafety conditions, their biological diversity was restored by passage through the organism of a guinea pig (causing agents of AD, TD, and APP) or suckling pigs (PCV-2). After that, the infectious potential of the strains was restored by three consecutive passages through monolayer subcultures of primary swine testicle (ST) cells/testicle cells of suckling piglets (for AD and TD viruses) or porcine alveolar macrophages (PAM, for PCV-2) to titers 6.5–7.5 $lgID_{50}$/ml. (NSC 'IECVM', 2020). The tubes' subcultures of ST cells were growth in Hanks BSS with conditioning by 0.01% yeast extract, 5–7% fetal calf serum (FCS, both Serva), and 0.5% lactalbumin hydrolysate (Sigma-Aldrich), 100 U/ml of penicillin, and 100 g/ml of streptomycin (Brovafarm Co, Kyiv), HIL-FCS. Mature cell monolayer ST subcultures and PAM were maintained with HIL without FCS and yeast extract under a temperature of 37 °C. Test bacteria

maintenance was conducted as recommended by the EU reference center (Sidoli and Pascucci, 1998). To obtain *Actinobacillus pleuropneumonia* test cultures in tubes, its museum suspension after pass per guinea pig organism (see above) was streaked on Petri's dish with chocolate agar supplemented with 1% blood-agar and incubated 24 hours at 37 °C. A suspension of daily bacterial culture was seeded on skew chocolate agar in test tubes (see below).

Studies of antimicrobial properties of the drug were conducted by Ten-concentration Dose Response Assay (TDRA) and Toxicity Assays (TA) taking into account the phenomenon of the dose responses hormesis (Calabrese, 2008) — *in vitro* and *in vivo*.

Briefly, for *in vitro* trials the approximately 20% of tested batch of the CVD 'NanoViroSan' (five random selected flaks) was separated into liquid and oil phases by low-speed centrifugation and liquid phase sterilized by microfiltration per disposable syringe cartridge with 0.3 μm-pores (Argos Technologies, India). In all these experiments, the liquid phase was used: ten concentrations were prepared from it in appropriate solvents (Table 3), which were also used as a blank (solvent control). For studies of virostatic activity cultural cells (ST or PAM) were seeded into cultural tubes (four ones on each drug dilution plus blank and mock cells without drug). After 2 hours of incubation at the 37 °C, ten different concentrations of the drug were added to each tube. ST cells and PAM were then infected with AD or TD viruses at MOI of 0.01 and with PCV-2 at MOI of 0.05, respectively. Mock (cells only with HIL-FCS) and Blank (cells with drug solvent in HIL-FCS) were viewed as positive and virus-infected cells only (HIL-FCS without drug and solvent) count as negative controls, respectively. Viral load was measured by Reed and Muench procedure: by cytopathic effects of the AD and TD viruses or the PCV-2 antigens presence by reaction of passive hemagglutinating (PHA), as prescribed in SOP of the NSC 'IECVM'. Compound cytotoxicity assay were carried out in the same fashion, except without viral infections of cell cultures. Bacteriostatic studies were conducted by analogous schedule, but instead of the cell cultures bacteriological mediums and reference bacteria by SOP of the NSC 'IECVM' were used. Namely, we used the APP agent in the final concentration of 4.5 lg bacterial cells, BC (by McFarland Standard, HiMedia Laboratories, India), and the bacteriological medium mentioned above. The total bacteria count (i. e. their viable concentration, TVC) of a sample was counted by the number of colony-forming bacterial units (CFU) in 1 ml sample at plate seed.

For *in vivo* trials the same control flaks were used, approximately 20% of the tested batch of the CVD 'NanoViroSan-L/O' (united five random selected flaks before separation into liquid and oil phases as above) and guinea pigs (weight 150–180 g each, four animals in each from three experimental groups — see Table 4).

On the 1st day of trials, animals of experimental group 2 were infected with agent AD: 1.2–1.5 cm^3 of the cultural suspension of AD virus (~ 3.0 lg $TCID_{50}$) in the lips of each animal of this group. Guinea pigs of group 3 were infected with agent APP: 1.2–1.5 cm^3 of the suspension of the agar culture of *A. pleuropneumonia* (~ 25,000 BC by McFarland's standard) intraperitoneally to each animal of this group. On the 2nd day of trials, there were three intramuscular doses (0.25, 0.50, and 1.50 ml, all injections in hind legs) and, as parallel, the untreated control was applied in all instances also. The course of treatment with the drug included the injections one the same dose three times with an interval of 48 hours in all instances. The results were recorded according to clinical signs with confirmation of the diagnosis by virological or bacteriological studies, respectively, according to the SOP of the NSC 'IECVM'.

Field tests. Groups of pedigree male piglets (Great White and Landrace breeds, age 3.5–4.2 months, n = 27) were kept in separate group cages (No. 1–5, see Table 2) of the sanitary facility of the experimental farm of the National Academy of Sciences of Ukraine. Each piglet was fed twice a day with 0.5–0.7 kg of complete grower feed with a protein content of 17–19%. In this group, serological and bacteriological studies, even before the introduction of the clinical protocol, showed asymptomatic carriage of the consortium of causative agents of circovirus infection (PCV-2), Aujeszky's disease (AD) and actinobacillary pleuropneumonia (APP). The clinical protocol of trials aimed to optimize the procedure for using the experimental drug 'NanoViroSan-L/O' according to Table 2.

Table 2 — Doses and schedule of 'NanoViroSan L/O' injections

Time, days	Group cages (experimental groups of pedigree male piglets)				
	1 (n = 5)	2 (n = 5)	3 (n = 7)	4 (n = 5)	5 (n = 5)
0	Forming of experimental groups by breed. Sampling.				
1–3	Subcutaneous injection of 0.5 ml three times a day	Subcutaneous injection of 0.5 ml once a day, three days in a row	Intramuscular injection of 0.5 ml three times a day	Intramuscular injection of 0.5 ml, once a day, three days in a row	Intramuscular injection of 2.0 ml, once a day, three days in a row
4–90	Clinical observations and sampling				

The subcutaneous injections (Subcutaneous, SQ) of the drug were made in the fold of skin between the ears and neck (the loose skin behind the ear of male pigs), intramuscular — in the muscles of the neck, closer to the incision line. Disinfection of the working surfaces of the machine and the skin of animals was carried out by 'Clinosan' according to the manufacturer's instructions (Ukrainian-Polish Joint Venture 'ZVK' LLC, Lviv, UA). Carrier status in pedigree piglet males on ADV, PCV-2, and APP agents was determined by traditional standard methods (according to SOP NSC 'IECVM'). Shots, in a week and a half and within three months from the beginning of the trials, the veterinary service of farms took samples of stabilized blood and semen by standard methods in a week (5–9 days), two (14–20 days), and three months (52–64 days). The preputial smears were taken with the application of the necessary hygiene-technological procedures that limit their microbiological contamination (Althouse and Lu, 2005). The same requirements were observed in the sampling of nasal swabs and stabilized (by EDTA) blood. All collected samples were stored at fridge temperature and brought to the laboratory in the next 8–12 hours. At the end of the experiment, in the course of breeding work, stamping out one or two young boars from the most defective in each experimental group in terms of exterior indicators was carried out. The tissue samples of the lungs, spleen, and brain were taken for laboratory investigations. Swine pox was diagnosed by identifying the lesions. Lesions were round to oval, usually 1.5–2.0 cm in diameter (Fig. 1). The brown to black crusts were seen after the vesicle stage. Histological diagnosis was conducted by identifying typical, large, intracytoplasmic inclusion bodies in cutaneous lesions (Hess et al., 2011).

Laboratory procedures. The field biomaterials were primarily evaluated in group samples-analogs by the PCR method in the laboratory for molecular epizootology. To determine the number of positive pigs in the experimental group, additional immunodiagnostic studies in other laboratories according to SOP of the NSC 'IECVM' were carried out. In short, target viruses (ADV and PCV-2) were detected in blood leukocytes, nasal and preputial samples, lymph nodes, spleen, lung, and brain tissues by virus isolation technique in primary trypsinized cell cultures with isolate identification by passive haemagglutination test (PHA), home-tests with using of appropriate commercial diagnostic tools. To positive samples, 1–2 consecutive passages through ST cells subcultures were applied (for AD virus) or PAM (for PCV-2); to negative samples — 3 consecutive passages. For serologic analyses of blood samples on target-viruses correspondent commercial ELISA kits (IDEXX, USA) were used. Isolation of target bacteria (*A. pleuropneumonia*) was done by seeding samples on nutrient chocolate agar supplemented with 1% blood agar The isolated bacteria, after Gram staining and oxidative/fermentative (OF) test, were identified by determining the phenotypic characteristics by means of biochemical series of bacteriological differentiation discs (HiMedia, India).

Statistical data processing was done at the level of 95% (ANOVA) with the help of the software Statistica 7 (StatSoft Inc., 1984–2004). Animal experimentation was conducted within standard ethical norms.

Final conclusion about the carrier status of pigs was a result of the full-format analysis of virologic, bacteriologic, and serologic data on each experimental group. A seropositive carrier animal was considered recovered if three months after the last administration of the target drug, the titer of antibodies in her blood samples against the investigated agent decreased by at least 3 dilutions compared to the initial (before remediation).

Results. Test data of the cytotoxic activity of the CVD drug 'NanoViroSan-L/O' against subcultures of the primary trypsinized culture of ST cells, as well as against viruses AD, TD, PCV-2 and the causative agent of APP are summarized in Table 3. After standard treatment of native and infected test systems (cell culture or bacteriological medium) with an active composite in the dose range from 0.001 to 10.00 mg/ml (by Methisazone), it was established that the drug 'NanoViroSan-L/O' exhibits clear dose-dependent effect. The cytotoxicity of the composite was registered only at its concentrations of 5.0 mg/ml and above (according to Methisazone). It was manifested by signs of cell necrobiosis: massive pycnosis of their nuclei at concentrations of 5–10 mg/ml on 3rd–4th days of incubation of test cultures (ST cells and PAM) and massive rounding and exfoliation of cells on 4th–6th days of incubation. Moreover, these signs appeared faster, the higher the concentration of the composite was in the range of 5–20 mg/ml for Methisazone.

According to the results of these tests using the culture of ST cells, the intensity of the reproduction of the Aujeszky's and Teschen disease viruses sharply decreased already at the concentrations of the composite of 0.1 and 0.5 mg/ml, respectively, and in no case was manifested in the presence of the drug in the concentration range of 1.0–1.5 mg/ml according to Methisazone (Table 3). 'Teschovirus-like dynamics' was observed in the patterns of reproduction of the reference strain of circovirus in a culture of porcine lung macrophages. In this series of tests, no cytopathic changes were registered in the cell culture, but the intensity of accumulation of the circovirus antigen in it, according to the results of the passive hemagglutination reaction, sharply decreased in the composite concentration range of 0.1–0.5 mg/ml and in no case manifested itself in the presence of the drug in the concentration range of 1.0–1.5 mg/ml according to Methisazone.

The data of a series of studies using the reference strain of the APP pathogen, shown in Table 3, indicate

the dose-dependence in vitro the antibacterial action of the 'NanoViroSan' composite too. The 1st signs of such action were already registered in the range of drug concentrations of 0.1–0.5 mg/ml according to Methisazone. However, complete inhibition of this agent reproduction occurred at doses of the drug of 1.0 mg/ml and higher.

Table 4 summarizes the primary data from *in vivo* biotoxicity and antimicrobial activity laboratory testing of the drug 'NanoViroSan-L/O' in guinea pigs (n = 48). The obtained data *in vivo* indicate a similar dose-dependence of the drug action as it has in vitro. At the same time, antimicrobial activity against both AD and APP agents was already recorded at dose of 0.5 mg/cm^3 at the level of complete protection of all guinea pigs (n = 8) from these diseases — as well as at a dose of 1.5 mg/cm^3. However, in guinea pigs, antibodies against the respective pathogens were detected on the 21st day after the last administration of the drug at a dose of 0.5 mg/cm^3 (n = 2 from 4), but was not at 1.5 mg/cm^3 (no one from 4).

In clinical trials under production conditions, a consortium of the ADV and PCV-2 viruses with the APP agent served as target infectious agents. The clinical and epizootological manifestation of the associated infection of pigs with the participation of this consortium in the experimental pig farm was an acute respiratory syndrome in piglets of the growing group with feed conversion at the level of 3.8–4.1, lethality up to 30% and culling up to 15%, as well as infertility of sows up to 10% and sporadically cases convulsions among suckling piglets and their death with pronounced opisthotonus, with up to 83% of nests viability during 45 days from birth. In pigs of a distinct group, up to 20% of individuals registered minor clinical signs of the PMWS syndrome complex and up to 2% — PDNS syndrome complex.

Table 3 — Summarized primary data on cytotoxic and antimicrobial activity of the drug 'NanoViroSan' *in vitro* (n = 40, p ≤ 0.03), details in text

Targets	Time, hrs	Dose-depend effect of drug's target composite in final concentration in cultural mediums (by Methisazone), mg/cm^3							
		0	0.01	0.1	0.5	1.0	1.5	5.0	10.0[***)]
ST cells[*)]	5	0000	0000	0000	0000	0000	0000	0000	0000
	24	0000	0000	0000	0000	0000	0000	0000	0000
	48	0000	0000	0000	0000	0000	0000	0000	0000
	72	0000	0000	0000	0000	0000	0000	0000	0000
	96	0000	0000	0000	0000	0000	0000	0000	0000
	120	0000	0000	0000	0000	0000	0000	00++	HHH+
	144	++++	++++	++++	++++	++++	++++	+H##	##HH
ADV[*)] in ST cells	5	0000	0000	0000	0000	0000	0000	n.d.	n.d.
	24	++++	0000	0000	0000	0000	0000	n.d.	n.d.
	48	++++	++++	0000	0000	0000	0000	n.d.	n.d.
	72	####	####	++++	0000	0000	0000	n.d.	n.d.
	96	-	-	##00	0000	0000	0000	n.d.	n.d.
TDV[*)] in ST cells	5	0000	0000	0000	0000	0000	0000	n.d.	n.d.
	24	++++	++++	0000	0000	0000	0000	n.d.	n.d.
	48	HHHH	++HH	00+H	H000	0000	0000	n.d.	n.d.
	72	####	####	00##	#000	0000	0000	n.d.	n.d.
	96	-	-	00--	H000	0000	0000	n.d.	n.d.
PCV-2[*)] in PAM	5	0%	0%	0%	0%	0%	0%	n.d.	n.d.
	24	25%	25%	25%	0%	0%	0%	n.d.	n.d.
	48	75%	75%	75%	0%	0%	0%	n.d.	n.d.
APP[**)] in bac. medium	24	25%	25%	10%	0%	0%	0%	n.d.	n.d.
	48	100%	100%	70%	25%	0%	0%	n.d.	n.d.
	72	100%	100%	100%	70%	0%	0%	n.d.	n.d.

Designations: [*)] '0000' — absence of cytopathic action in any tubes; '++++' — destruction to 25% of monolayer in all 4 tubes; *'HH ##'* — destruction to 50% and more than 75% of monolayer in 2 tubes, respectively and presence of according agent's antigens; '0000' — presence of PCV-2 antigens without of cytopathic effect: 25–75%, volumes of cells from the total area of their monolayer that were positive for PCV-2 antigen in the immunoperoxidase test; [**)] 25% — to 30 colonies; 70% — to 60 colonies; 100% — > 60 colonies; [***)] rest checkered two concentrations (15 and 20 mg/ml) not show as were significantly more cytotoxic; n.d. — not did.

Table 4 — Summarized results of the 'NanoViroSan' toxicity evaluation and antimicrobial activity trial against ADV and APP agent in guinea pigs-model ($p \leq 0.01$; n = 48)

Experimental groups (agents load)	Time, days	Disease signs appearance after 'NanoViroSan' injections in doses, mg/cm^3:			
		0	0.25	0.50	1.50
1 — Guinea pigs without agents load, n = 16	1–90	'0' (n = 4)	'0' (n = 4)	'0' (n = 4)	'0' (n = 4)
2 — ADV (~ 3.0 lg $TCID_{50}$ on animal, intradermally), n = 16	1	'0' (n = 4)	'0' (n = 4)	'0' (n = 4)	'0' (n = 4)
	7	'0' (n = 1), '+' (n = 3)	'0' (n = 1), '+' (n = 3)	'0' (n = 4)	'0' (n = 4)
	14	'+' (n = 1), '+++' (n = 3)	'+' (n = 4)	'0' (n = 4)	'0' (n = 4)
	21	'+++' (n = 1), '#' (n = 3)	'+' (n = 1), '+++' (n = 3)	'0' (n = 4)	'0' (n = 4)
3 — APP (~ 5.0 lg BC on animal, intraperitoneally), n = 16	1	'0' (n = 4)	'0' (n = 4)	'0' (n = 4)	'0' (n = 4)
	7	'#' (n = 4)	'0' (n = 4)	'0' (n = 4)	'0' (n = 4)
	14	-	'0' (n = 1), '+' (n = 3)	'0' (n = 4)	'0' (n = 4)
	21	-	'+++' (n = 2), '#' (n = 2)	'0' (n = 4)	'0' (n = 4)

Designations: '0' — absence of any morbidy reaction; **'+'** — induration at the drug injection site for ADV and depression for APP; **'+++'** — itching and necrosis of the skin at the injection site for ADV and bloody nasal discharge and exhaustion for APP; '#' — death with typical disease pathomorphology.

So, pedigree male piglets, affected under the conditions of commercial pig farming by the above-mentioned associative microflora, were treated with the target composite drug according to five experimental schemes, which is aimed at optimizing the use of 'NanoViroSan-L/O' according to the method of administration and dose (Table 2). The obtained data are summarized in Table 5.

Table 5— Trial of 'NanoViroSan' activity on drift of male pigs, n = 43; $p \leq 0.023$ (by summary tests results — see text)

Time, days	Positive carrier status of experimental pedigree male piglets on Pseudorabies virus / Porcine Circovirus Genotype 2 / *A. pleuropneumonia*, animal number in subgroups					
	1 (n = 5)	2 (n = 5)	3 (n = 7)	4 (n = 5)	5 (n = 5)	Mock (n = 16)
0	3/3/4	4/2/2	5/3/3	2/4/5	5/3/3	9/12/14
≥ 7	0/3/4	2/0/2	0/0/0	0/0/0	0/0/0	-
≥ 14	0/0/-	0/0/-	0/1/-	0/1/-	0/0/-	-
≥ 21	2/1/1	1/1/0	2/1/0	0/0/0	0/0/0	11/14/9
≥ 35	2/2/0	-	-	0/0/0	0/0/0	-
≥ 90	2/2/3	2/1/0	1/2/0	0/0/0	0/0/0	14/14/7

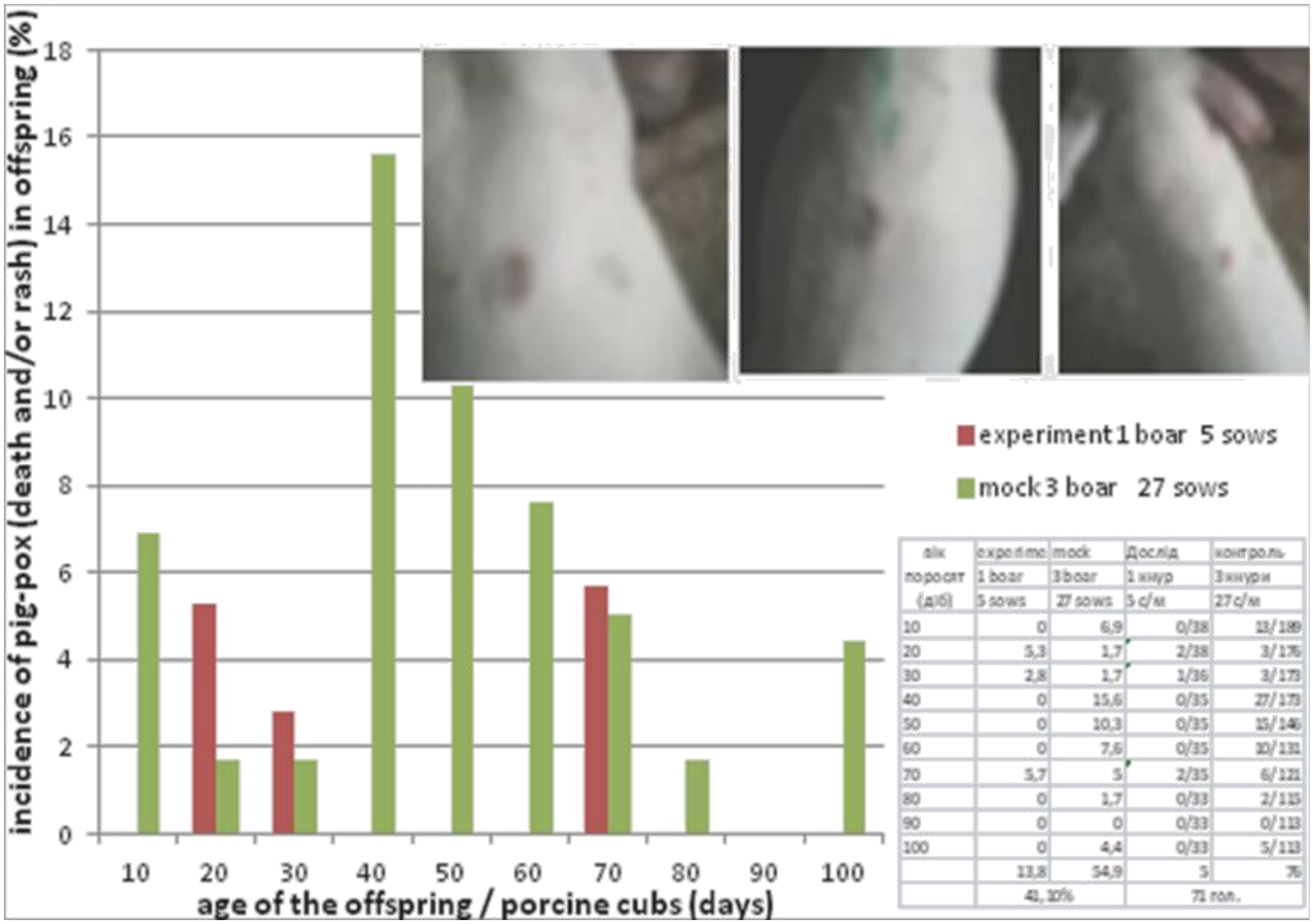
Description: Groups No. 1–5: piglets 45-day-old age accordingly to Table 2; Mock: pedigree male piglets with the same status but without 'NanoViroSan' covering (was applicated a Amoxicillin protocol); '0/0/0' — absence all of target agents; '2/0/0' — presence of PCV-2 in two pedigree male piglets and absence all other target agents in all piglets of this experimental group; '-' — test not done.

During the 3-month research, no signs of adverse effects of the drug 'NanoViroSan L/O' on pigs, under the conditions of traditional Ukrainian pig farming, were registered. There no clinical signs of AD, PCV-2, and APP were registered during the entire period of observation among experimental animals aged 4.5 months, treated intramuscularly three times, once a day, for three consecutive days in a row in doses of 0.5 (group No. 4) and 1.5 cm^3/20 kg (group No. 5). In the piglets of the remaining experimental groups treated intramuscularly three times in a dose of 0.5 cm^3/20 kg with a minimum interval (3–4 hours, group No. 3), virus-carrying of AD and PCV-2 agents did not stop until the end of the 3-month experiment. At the same time, the carriering of the APP agent in piglets of group No. 3 was treated already on the seventh day after one-day three-time intramuscular treatment with an interval of 3–4 hours and was not restored until the end of the experiment. At the same time, viral carriage of AD and PCV-2 agents in this group stopped in a 2-week period after sanitation and then resumed In the groups of subcutaneous injection of the drug, the carriage of the pathogen of APP stopped only in piglets covered with drug three times with the highest interval, i.e. of 14–18 hours (group No. 2), and the virus carriage of the AD and PCV-2 in this group almost did not stop. The final results of the trials of three times subcutaneous administration with minimal intervals (group No. 1) were the same as in the control group of piglets (see Mock in Table 5).

In order to confirm the obtained results of laboratory and clinical studies, given above, at the initial and final stages of the studies, a retrospective analysis of the data of serological studies and herd zootechnical scoring was carried out. According to the results of this analysis, three months after the last administration of the drug, antibodies against the causative agents of AD and APP were detected in piglets groups of No. 2–5, but their antibodies against the causative agent of circovirus

infection were not detected in groups of piglets No. 4 and No. 5. Titers of antibodies against PCV-2 in groups No. 2 and No. 3 (dose of 0.5 ml, but different schemes of its use) during this period fell, on average, from 1:64.2 ($n = 5$, SD = 52.3%, $p < 0.05$) and 1:56.3 ($n = 5$, SD = 81.2%, $p < 0.05$) to 1:14.4 ($n = 5$, SD = 31.7%, $p < 0.03$) and 1:16 ($n = 5$), SD = 28.1%, $p < 0.02$), respectively. At the same time, during the test period (more than 3 months), the piglets of the 3rd, 4th, and 5th groups in terms of average live weight during control weighing exceeded the mock-piglets ($n = 16$) by 337 ± 57 g ($n = 7$), 1,220 ± 89 g ($n = 5$) and 1,170 ± 44 g ($n = 5$), respectively. In pigs of group No. 1 (a dose of 0.5 ml for a one-day subcutaneous injection three times a day), titers of antibodies against PCV-2 during the 3-month trials practically did not change: 1:67.6 ($n = 5$, SD = 85.2%, $p < 0.05$) versus 1:77.5 ($n = 5$, SD = 73.8%, $p < 0.05$), respectively. There was no difference in the average weight of the animals of this group ($n = 5$) and mock piglets ($n = 16$).

Fig. 1 shows the results of testing the 'NanoViroSan' composite in a commercial pig farm for the eradication of swine pox in young animals. On the eve of the mating campaign and at the beginning of its implementation, one of four breeding boars and 5 of 32 sows from the nucleus of the herd were intramuscularly covered twice at the rate of 1 ml of the drug per 20 kg of body weight with an interval of 12–14 hours. In the course of the mating campaign and the entire next gestation period, no deviations in the state of health from the physiological norm were registered in the 'NanoViroSan' covered animals. After the birth of piglets ($n = 38$ in the experimental group and $n = 189$ in the control group, s. born from non-inoculated sows and boars), morbidity (by indicators of viability and skin rashes) was carried out by the staff of the pig farm.



вік поросят (діб)	experime 1 boar 5 sows	mock 3 boar 27 sows	Дослід 1 кнур 5 с/м	контроль 3 кнури 27 с/м
10	0	6,9	0/38	13/189
20	5,3	1,7	2/38	3/176
30	2,8	1,7	1/36	3/173
40	0	15,6	0/35	27/173
50	0	10,3	0/35	15/146
60	0	7,6	0/35	10/131
70	5,7	5	2/35	6/121
80	0	1,7	0/33	2/115
90	0	0	0/33	0/113
100	0	4,4	0/33	5/113
	13,8	54,9	5	76
	41, 10%		71 гол.	

Figure 1. The results of treatment with the drug 'NanoViroSan' of the pig herd nucleus to prevent swine pox in the offspring (see the text for more details).

Unfortunately, this period coincided with the events of martial law, which were not compatible with conducting other, more evidential studies. However, the obtained data confirmed the significant effectiveness of the drug 'NanoViroSan' for the prevention of swine pox in commodity farms ($n = 227$, $p < 0.05$). A feature of the therapeutic effect of the drug on piglets with pox was an increase in the size of individual papules as a result of its edema (cold painless edema, without loss of appetite and positive dynamics of feed conversion) in the first

5–7 days after the last (third) administration at a dose of 0.5 cm^3/20 kg. Within 1.5–2.5 weeks, the animals fully recovered without additional interventions (n = 12), and when applied externally with zinc ointment — after 3–5 days (n = 8).

Based on the analysis of the obtained results and the experience of using 'Lipozone' (a drug-analog), the composite drug 'NanoViroSan-L/O' can be recognized as a universal drug and recommended for further trials in the current order for viral, bacterial, and mixed infections of pigs in dose 1.5–2.0 ml to adult pigs of 20 kg body weight intramuscularly three times with an interval of up to 24 hours, if necessary — repeat the scheme after a week or a half; for young pigs from 15 days of age — the same, but in a dose of 1.0 ml per 10 kg of weight.

Discussions and conclusions. An urgent problem of domestic pig farming is its high dependence, due to a number of diverse reasons, on the intensive use of a wide range of antibiotics (Linciano et al., 2019). For the past 40 years, β-lactam antibiotics have been the most sought-after veterinary support for animal husbandry — due to their wide spectrum of action, market availability and ease of use. However, their irresponsible use in poultry farming, animal husbandry, fish farming, etc., has caused global problems for public health — due to the emergence of resistance genes (ARGs) in pathogenic human bacteria.

Therefore, the priority task in this direction is the search and development of potentially clinically effective drugs alternative to these antibiotics, as priority (Linciano et al., 2019). Therefore, in the development of the target drug's antibacterial constituent, special attention was paid to search for inhibitors of microbial enzymes, the activity of which is related to zinc-containing coenzymes. These enzymes are deprived of a wide range of species of pathogenic bacteria and fungi to survive in adverse conditions, in particular in the presence of antibiotics. Such enzymes include β-lactamases of pathogenic staphylococci, *Klebsiella*, and enterobacteria. Fig. 2 (by Kim et al., 2013 — 'bell-shaped' structure in the center of the enzyme molecule) shows an example of the zinc-containing coenzyme of the most dangerous bacterial β-lactamase of the type 'New Delhi' NDM-1 (Kim et al., 2013). Other microbial enzymes possess similar zinc-containing coenzymes - for example, carbonic anhydrases (CA, EC 4.2.1.1) of gonorrhea pathogens *Neisseria gonorrhoeae*, cholera *Vibrio cholerae*, 'American trypanosomosis' (Chagas' disease) *Trypanosoma cruzi*, pathogenic fungi *Cryptococcus neoformans*, *Candida glabrata* and *Malassezia globosa* (Supuran, 2021).

According to numerous data from the world literature, active inhibitors of these microbial enzymes are compounds of the chemical class of isatin-b-thiosemicarbazones (IBT). The thiol group of these compounds (see in circle of Fig. 3) forms various coordination bonds with the zinc ions of the active site of the zinc-containing coenzymes as above — i.e. neutralizing of their (Li et al., 2021).

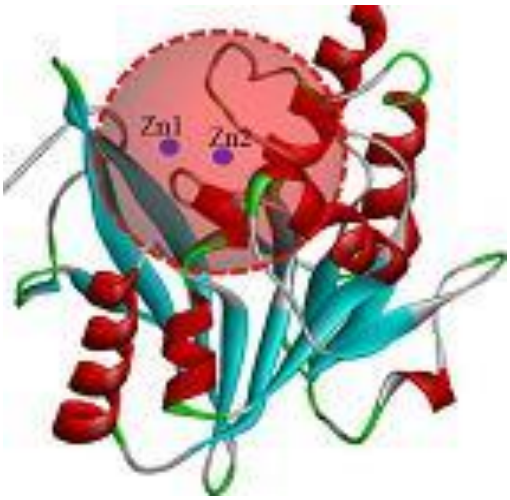


Figure 2. Structural model of the bacterial enzyme that 'kills' β-lactam antibiotics (details in text).

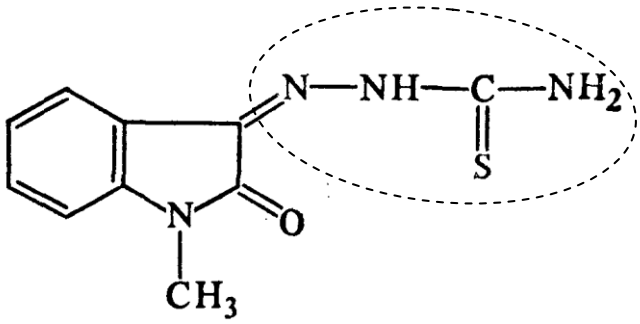


Figure 3. Structural formula of Methisazone.

For example, the highest level of inhibition effect of β-lactamases of methicillin-resistant *Staphylococcus aureus* (MRSA) by mentioned IBT-compound was showed in it's inhibitory concentration of IC50 ≤ 10 μM (Zhang et al., 2015). The specified concentration, according to the international database on the toxicity of chemical compounds, is 1,500 times lower than the index of toxic action of Methisazone, the active substance of which belongs to IBT class of chemical compounds namely (BV-BRC, 2022). Methisazone has been known since the 1950s as a virostatic agent with high therapeutic and prophylactic efficacy against smallpox in humans and currently, it is considered a domestic antiviral drug with a certain antibacterial effect (Lozjuk et al., 1996). It is well known that the antiviral activity of this drug is caused by blocking the expression of early proteins (Patskovsky et al., 1996) of many species of both DNA- and RNA-containing viruses (Levinson, 1975; Andreani et al., 1977, Bauer et al., 1970). Therefore, we chose Methisazone as a promising drug for the creation of a

composite drug with targeted action. To increase the activity of the target composite against bacteria and expand the antiviral spectrum, we applied the additional capabilities of Silgeran, the new drug of domestic origin.

The circumstances of the martial law in Ukraine did not allow us to conduct more detailed studies of the effectiveness of the drug — in particular, in a comparative aspect with analogous drugs of domestic and foreign production (i.e. conduct trial on therapeutically bioequivalence). However, the composition of the composite drug 'NanoViroSan' contains the medicals that have been obligatorily studied by its manufacturers — both at the preclinical and clinical levels during its state registration. That is, if the main task of the current tests is to study the compatibility of the components and the preservation of their therapeutic properties in the composite under production conditions, then the results obtained at a sufficient level of probability show a positive decision, provided that certain requirements are met regarding the method of use of the drug.

The obtained results of preclinical and clinical tests testify to the pronounced activity of the drug designed by us against the causative agents of Aujeszky's and Teschen diseases, type 2 circovirus, pig-pox as well as the causative agent of swine actinobacillary pneumonia. Its three-time intramuscular application made it possible to recover the pigs from carrying the specified pathogens. It is known that breeding boars are more resistant than other production groups to the clinical manifestation of infectious diseases — even to ASF (Bisimwa et al., 2021) and therefore, as a rule, serve as a hidden source of infectious agents. Moreover, from our unpublished data, the 8 sows that we treated orally with this drug until stamping out (about 9 days) were healthy in the presence of all other untreated pigs in the ASF outbreak No. 428/2018 (SSUFSCP, 2022).

Therefore, the obtained results indicate the promising news of the injectable composite drug 'NanoViroSan' as a possible multipurpose agent in pig breeding. This meets the requirements of the modern world trend of medicinal chemistry for the development of multi-target ligands (MTDL), which is gradually changing the traditional methodological approach of 'one drug — one target' (Hashmi et al, 2021). Due to the harmonious combination of active substances in the drug, mainly antiviral (Methisazone, nanoxide of zinc) and antibacterial (Silgeran) direction, this complex remedy can be useful for use in emergency epizootic situations. For the bio protection of pig farming under the conditions of martial law in Ukraine, it is expedient to test the drug in enzootic focuses of ASF, in particular in small-scale and homestead farms that are located in zones of ASF spreading among wild boar population. According to our hypothesis, low virulent variants of the ASFV causative agent circulate in these zones as part of viral-bacterial associations (Buzun and Kolchyk, 2022). Summarizing, it is possible to predict with high probability that the complex use of composite preparations with probiotics, medical serums, and vaccines (primarily inactivated, from local strains of pathogens) will allow pig farming to quickly get rid of the current level of antibiotic load. In turn, this will significantly increase the agricultural export potential of Ukraine, as an influential participant in the fight against global hunger.

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