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FORMATION OF INTESTINAL BACTERIOCENOSIS IN CALVES ASSOCIATED WITH BACTERIAL INSEMINATION OF THE UTERINE CANAL IN HIGH-YIELDING COWS

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Summary. The article provides information on the microbiota composition in the vaginal secretions of cows during the dry period, specifically 30–15 days before calving. The article discusses how endometritis affects the qualitative and quantitative composition of the conditionally pathogenic microflora, as well as how dysbiotic microbiota disorders in the birth canal impact the development of gastrointestinal diseases in calves. The study results establish the quantitative limits of the conditionally pathogenic microbiota, which requires correction when exceeded. When cows have dysbiotic changes, there is an increase in the number of certain microorganisms in their vaginal swabs. Specifically, the number of *Escherichia coli*, sulfite-producing clostridia, and saprophytic microorganisms of the genus *Bacillus* goes beyond 6.0 ± 0.1 lg CFU/cm^3, while staphylococci and yeast-like fungi of the genus *Candida* exceed 5.9 ± 0.1 lg CFU/cm^3. Additionally, calves born to cows with dysbiotic disorders of vaginal secretion had dysbiotic disorders in the gastrointestinal tract in 86.1% of cases. In these cases, the number of lactobacilli was not higher than 4.8 ± 0.1 lg CFU/cm^3, bifidobacteria — 4.7 ± 0.1 lg CFU/cm^3. The number of *Escherichia coli* exceeded 4.6 ± 0.1 lg CFU/cm^3, which in 48.4% of cases resulted in the development of diarrhea of varying severity

Keywords: microbiota, dysbiosis, normal intestinal microflora, normal vaginal flora

Introduction. There is a lack of clear data regarding the development of intestinal bacteriocenosis in calves in relation to the bacterial contamination of the genital tract of highly productive cows (Bentsa, 2018; Bortnichuk, Sadovskyi and Sorokina, 1997; Basova et al., 2016; Maslianko et al., 2013; Kalinichenko, Korotkykh and Tishchenko, 2016; Petrova and Domracheva, 2012; Stravskyi and Stravska, 2014; Vlizlo, 2012; Yakubchak et al., 2005; Garrity et al., 2005; Hadzevych et al., 2021; Diao, Zhang and Fu, 2019; Liu et al., 2019; Bi et al., 2019; Alipour et al., 2018; Mayer et al., 2012; Uyeno, Sekiguchi and Kamagata, 2010; Baldwin et al., 2004).

Previous studies have shown that the number of microorganisms present in calves is minimal up to three days of age. The quantity of bifidobacteria and lactobacilli does not exceed 3 lg colony forming units per 1 cm^3 of large intestine content (Hadzevych et al., 2021).

After 15 days of age, calves develop a more stable gastrointestinal microflora that includes a protective barrier known as colonization resistance. The microflora in the intestinal mucosa displays antagonistic activity, preventing the penetration of pathogens. However, in newborn calves, the intestinal microflora's qualitative and quantitative composition is not yet strong enough to prevent the colonization of foreign microorganisms, including pathogens.

Numerous studies have shown that the microbiota of the birth canal plays a leading role in the formation of the microbiome of newborns (Bortnichuk, Sadovskyi and Sorokina, 1997; Basova et al., 2016 Maslianko et al., 2013; Kalinichenko, Korotkykh and Tishchenko, 2016; Petrova and Domracheva, 2012; Stravskyi and Stravska, 2014).

The aim of the study was to investigate the formation of calves' intestinal bacteriocenosis in relation to the bacterial colonization of the birth canal of highly productive cows.

Materials and methods. The study was carried out in 2022 in the Laboratory of Animal Bacterial Diseases of the NSC 'IECVM'. To determine the normocenosis, the vaginal secretion of cows in the dry period 15–30 days before calving and the swabs from the intestinal tract of calves at the age of 7–10 days were examined. All data were recorded, and at the end of the observation period, the data were analyzed and conclusions were drawn. A total of 120 cows and 114 calves were studied. The animal studies were conducted in accordance with the basic principles of bioethics.

Animals were housed, maintained, and fed according to standards and rations. The quantitative and qualitative diets of animals in different housing systems did not differ. The material was delivered to the laboratory and examined within 2 hours of collection. Serial 10-fold dilutions of homogenized material in a sterile isotonic sodium chloride solution from 10^{-1} to 10^{-10} were performed to determine the quantitative value of microorganisms.

From each tube of the titration series, 1 cm^3 of homogenate was inoculated onto the optimal culture media for each microbial species and incubated under optimal temperature conditions and hours.

Simple and selective culture media produced by Farmaktiv LLC (Ukraine) and HiMedia Laboratories Prv. Limited (India) were used. For the isolation of enterobacteria, Endo, Ploskirev, Levin, MacConkey media, bismuth sulfite agar, Olkenitsky medium, selenite broth (for the accumulation of *Salmonella*), Simons medium were used; for staphylococci — egg yolk salt agar, Chistovich's medium; for streptococci — media containing glucose (1%), blood (5–10%) and serum (10–20%); for fungi and yeasts — Sabouraud's and Wort's agar; for anaerobes — Kitt-Tarozzi, Wilson-Blair agar, L. D. agar with esculin (for anaerobes), Voget-Fredette agar. For the isolation of bifidobacteria, Blaurock's medium was used, and for the isolation of lactobacilli, LactoBacAgar.

To determine the hemolytic activity of microorganisms, 5% blood agar was used, and coagulase activity was determined using dry rabbit citrate plasma produced by Pharmstandard-Biolik PJSC (Ukraine).

After incubation of the cultures on the media under optimal conditions, the colonies grown from each dilution were counted. The population level of microorganisms was expressed in the decimal logarithm of the indicator — lg CFU/cm^3 (colony forming units in 1 cm^3 of material).

To determine the number of microorganisms, the degree of dilution, the number of growing colonies and the inoculation dose were taken into account.

The number of colonies of forming units in 1 cm^3 of material was calculated using the formula (1):

$$M = \frac{N}{V} \times 10^{n+1} \quad (1)$$

where: M is the number of microorganisms in 1 cm^3; N is the average number of colonies in 1 bacteriological dish; V is the volume of suspension applied to the surface of the agar during seeding; 10^{n+1} is the dilution from which seeding was performed (Vlizlo, 2012; Yakubchak et al., 2005). Bacteria were identified according to Bergey's Manual of Systematics Bacteriology (Garrity et al., 2005).

Statistical processing of numerical data was carried out by the method of alternative analysis using the application Microsoft Excel (Microsoft Office). The relative share of the characteristic in the statistical population (in percent) (M) and its error (m) were determined, and the level of reliability (p) was determined.

Results. The results of the determination of the composition of the microflora of the vaginal secretion of cows in the dry period are shown in Table 1.

Table 1 — Microbiota of vaginal secretion of cows in the dry period 15–30 days before calving

Indicators	Frequency of isolation of microorganisms from cows that							
	had no clinical manifestations of diseases of the reproductive system (n = 80)				had clinical manifestations of diseases of the reproductive system (n = 40)			
	n	%	colonization density, lg	amount, in 1 cm^3	n	%	colonization density, lg	amount, in 1 cm^3
Lactobacilli	80	100	5.3 ± 0.1	989 ± 238×10^4	32	80	2.4 ± 0.1	15 ± 4.9×10^2
Escherichia coli without hemolytic properties	58	72.5	2.4 ± 0.1	212 ± 51.2×10^2	40	100	6.0 ± 0.1	68 ± 17.4×10^5
Escherichia coli with hemolytic properties	2	2.5	1 ± 0	4 ± 1.5×10^1	25	62.5	4.3 ± 0.1	179 ± 51×10^3
Sulfite-reducing clostridia	2	2.5	1 ± 0	35.8 ± 23×10^1	22	55	6.0 ± 0.1	100 ± 26×10^5
Representatives of the family Enterobacteriaceae	80	100	4.0 ± 0.1	86 ± 39×10^3	40	100	7.3 ± 0.1	184 ± 43×10^6
Enterococci	35	43.7	2.4 ± 0.1	91 ± 31×10^2	34	85	4.8 ± 0.1	78 ± 25×10^4
Staphylococci coagulase-negative	80	100	2.3 ± 0.1	126 ± 28×10^3	40	100	5.9 ± 0.1	105 ± 40.4×10^5
Staphylococci coagulase-positive	0	0	0	0	28	70	5.1 ± 0.1	176 ± 52.7×10^4
Streptococci	80	100	3.5 ± 0.1	35 ± 11.2×10^3	40	100	6.3 ± 0.1	411 ± 115×10^5
Yeast-like fungi of the genus *Candida*	56	70	4.0 ± 0.1	104 ± 39.3×10^3	23	57.5	5.9 ± 0.1	152 ± 54.7×10^5
Saprophytic microorganisms of the genus *Bacillus*	16	20	3.7 ± 0.1	22 ± 3.8×10^3	32	80	6.8 ± 0.1	152 ± 44.7×10^6

In cows with endometritis, a violation of the qualitative ratio of microflora was observed due to the appearance and increase in the vaginal secretion of *Escherichia coli*, staphylococci and sulfite-reducing clostridia, the appearance of hemolytic strains of *Escherichia coli* and coagulase-positive staphylococci, and a decrease in lactobacilli:

(1) the amount of lactobacilli with endometritis was $15 \pm 4{,}9\times10^2$ CFU/cm^3, in clinically healthy animals — $989 \pm 238\times10^4$ CFU/cm^3;

(2) the amount of *Escherichia coli* — $68 \pm 17{,}4\times10^5$ CFU/cm^3, in clinically healthy animals — $212 \pm 51{,}2\times10^2$ CFU/cm^3;

(3) the amount of sulfite-reducing clostridia — $100 \pm 26\times10^5$ CFU/cm^3; in clinically healthy animals — $35{,}8 \pm 23\times10^1$ CFU/cm^3;

(4) the amount of bacteria from the family Enterobacteriaceae to $184 \pm 43\times10^6$ (in clinically healthy animals — $86 \pm 39\times10^3$ CFU/cm^3;

(5) the amount of yeast-like fungi of the genus *Candida* to $152 \pm 54{,}7\times10^5$ CFU/cm^3; in clinically healthy animals — $104 \pm 39{,}3\times10^3$ CFU/cm^3;

(6) the amount of saprophytic microorganisms of the genus *Bacillus* to $152 \pm 44{,}7\times10^6$ CFU/cm^3; in clinically healthy animals — $22 \pm 3{,}8\times10^3$ CFU/cm^3;

(7) the amount of coagulase-positive staphylococci — $176 \pm 52{,}7\times10^4$ CFU/cm^3, in clinically healthy animals — $126 \pm 28\times10^3$ CFU/cm^3.

Calves obtained from cows with endometritis in 86.1% of cases had dysbiotic disorders in the gastrointestinal tract (Table 2), namely the number of lactobacilli was not higher than 4.8 ± 0.1 lg CFU/cm^3, bifidobacteria — 4.7 ± 0.1 lg CFU/cm^3. The number of *Escherichia coli* was 6.2 ± 0.1 lg CFU/cm^3 (Table 3), saprophytic microorganisms of the genus *Bacillus* — 6.0 ± 0.1 lg CFU/cm^3, which in 48.4% of cases (15 heads) resulted in the development of diarrhea of varying severity.

Table 2 — Presence of dysbiotic changes in the gastrointestinal tract of calves from sick and healthy cows

Calves from healthy cows (n = 78)				Calves obtained from cows with endometritis (n = 36)			
no dysbiotic changes in the gastrointestinal tract		had dysbiotic changes in the gastrointestinal tract		no dysbiotic changes in the gastrointestinal tract		had dysbiotic changes in the gastrointestinal tract	
n	%	n	%	n	%	n	%
68	87.1	10	12.9	5	13.9	31	86.1

Table 3 — Results of studies of rectal swabs from calves of 5–7 days of age

Indicators	Qualitative and quantitative changes in the microbiota in the rectum of calves							
	without dysbiotic changes (n = 73)				with dysbiotic changes (n=41)			
	n	%	colonization density, lg	amount, in 1 cm^3	n	%	colonization density, lg	amount, in 1 cm^3
Lactobacilli	73	100	8.0 ± 0.1	$143 \pm 40\times10^7$	41	100	4.8 ± 0.1	$45 \pm 9.8\times10^4$
Bifidobacteria	73	100	6.8 ± 0.1	$96 \pm 38\times10^6$	41	100	4.7 ± 0.1	$48 \pm 14\times10^4$
Escherichia coli	73	100	4.6 ± 0.1	$83 \pm 20\times10^4$	41	100	6.2 ± 0.1	$182 \pm 38\times10^5$
Sulfite-reducing clostridia	15	20.5	2.7 ± 0.1	$44 \pm 6\times10^2$	22	53.6	4.8 ± 0.1	$65 \pm 15\times10^4$
Conditionally pathogenic microorganisms from the family Enterobacteriaceae	73	100	2.8 ± 0.1	$114 \pm 50\times10^2$	41	100	6.1 ± 0.1	$116 \pm 32\times10^5$
Enterococci	73	100	3.8 ± 0.1	$230 \pm 67\times10^3$	41	100	5.8 ± 0.1	$49 \pm 16\times10^5$
Staphylococci	60	82.1	3.5 ± 0.1	$135 \pm 54\times10^3$	23	56.1	4.6 ± 0.1	$113 \pm 82\times10^4$
Saprophytes of the genus *Bacillus*	73	100	2.9 ± 0.1	$223 \pm 45\times10^2$	41	100	6.0 ± 0.1	$137 \pm 46\times10^5$

Discussions. The results of the research once again confirmed the data obtained in previous years that in calves, in order to exclude dysbiotic disorders, it is necessary to maintain the optimal composition and quantitative level of the main microflora of the intestinal tract, in particular, the number of lactobacilli should not be lower than 6 lg CFU/cm^3, bifidobacteria — 7 lg CFU/cm^3, the number of *Escherichia coli* not higher than 7 lg CFU/cm^3 (Hadzevych et al., 2021). In addition, it has been proved that the presence of a violation of the qualitative and quantitative composition of the microflora in the birth canal in cows contributes to the occurrence of dysbiotic changes in the microflora of the gastrointestinal tract of calves in 86.1% of cases and in 48.4% of cases of diarrhea of varying severity. Based on the analysis of literature data, no unified normative indicators for the composition of the gastrointestinal tract normoflora in calves and vaginal secretions in cows

have been established (Bortnichuk, Sadovskyi and Sorokina, 1997 Basova et al., 2016 Maslianko et al., 2013; Kalinichenko, Korotkykh and Tishchenko, 2016; Petrova and Domracheva, 2012; Stravskyi and Stravska, 2014; Vlizlo, 2012; Yakubchak et al., 2005; Garrity et al., 2005; Hadzevych et al., 2021; Diao, Zhang and Fu, 2019; Liu et al., 2019; Bi et al., 2019; Alipour et al., 2018; Mayer et al., 2012; Uyeno, Sekiguchi and Kamagata, 2010). However, there is undeniable evidence that dysbiosis and opportunistic microbiota in the birth canal play a significant role in the etiology and development of endometritis, along with specific infections (Bortnichuk, Sadovskyi and Sorokina, 1997). The microbiota of the gastrointestinal tract of calves after birth is formed within seven days (Hadzevych et al., 2021; Diao, Zhang and Fu, 2019), according to the results of some authors — twelve days (Uyeno, Sekiguchi and Kamagata, 2010). There are reports that microbial colonization of the mammalian intestine can begin even before birth, but these observations are controversial due to problems with reliable sampling and analysis of the rarely distributed microbiota. The mammalian gastrointestinal tract is populated with a diverse microbiota before birth, which changes rapidly in early postnatal life (Alipour et al., 2018). The method and regimens of feeding also influenced the direct transmission of bacteria from the mother and the environment to newborns. In bottle-fed animals, bacteria from the mother's vagina (46%), ambient air (31%), and floor (12%) predominated (Bi et al., 2019). In addition, there are studies indicating that certain microflora may be genetically or epigenetically influenced. Understanding that there are several correspondences between the intestinal microbiota of healthy calves and that there may be a genetic influence on the fecal flora may help prevent diarrheal diseases in the future (Mayer et al., 2012). In diseases of the reproductive organs of cows, potentially pathogenic bacteria contaminate calves at birth and contribute to the development of dysbiotic states — persistent qualitative and quantitative changes in the microbiota and gastrointestinal diseases (Yakubchak et al., 2005; Garrity et al., 2005; Hadzevych et al., 2021).

According to the literature, the reproductive system of cows is considered infected in the absence of bifidobacteria and lactobacilli in the cervical canal and the presence of opportunistic microflora, especially with pronounced hemolytic properties in the amount of 2 lg CFU/cm^3, or without hemolytic properties in the amount of 4 lg CFU/cm^3, or the presence of opportunistic microflora in associations consisting of 2 or more species, especially if they have pronounced virulent and antibiotic-resistant properties (Petrova and Domracheva, 2012). It has been reported that the microflora of the genital tract gradually increases during gestation regardless of breed, especially the number of *Escherichia coli* (Liu et al., 2019). Our studies show that the maximum amount of saprophytic microorganisms of the genus *Bacillus* in vaginal swabs 15–30 days before calving should not exceed 3.7 ± 0.1 lg CFU/cm^3, staphylococci — 2.3 ± 0.1 lg CFU/cm^3, yeast-like fungi of the genus *Candida* — 4.0 ± 0.1 lg CFU/cm^3, *Escherichia coli* without hemolytic properties — 2.4 ± 0.1 CFU/cm^3, sulfite-producing clostridia — 1.0 ± 0.1 lg CFU/cm^3, representatives of the family Enterobacteriaceae — 4.0 ± 0.1 lg CFU/cm^3. Other qualitative and quantitative indicators of the microbiota need to be analyzed and, if necessary, corrected with probiotic, antibacterial drugs or improved animal housing conditions.

Calves born from cows with dysbiotic disorders of vaginal secretion in 86.1% of cases had dysbiotic disorders in the gastrointestinal tract, namely the number of lactobacilli 4.8 ± 0.1 lg CFU/cm^3, bifidobacteria — 4.7 ± 0.1 lg CFU/cm^3, *Escherichia coli* — 6.2 ± 0.1 lg CFU/cm^3, saprophytic microorganisms of the genus *Bacillus* — 6.0 ± 0.1 lg CFU/cm^3, which in 48.4% of cases resulted in the development of diarrhea of varying severity.

Conclusions. 1. Excessive accumulation of opportunistic microbiota in the birth canal leads to the development of dysbiosis and endometritis in cows and contributes to the occurrence of gastrointestinal disorders in calves at birth.

2. Qualitative and quantitative changes in the microbiota of vaginal secretions were observed in cows with endometritis. Hemolytic forms of *Escherichia coli* were found in 62.5% of cases, sulfite-producing clostridia in 55%, and coagulase-positive staphylococci in 70%. No particularly dangerous specific pathogens were found.

3. Quantitative limits of conditionally pathogenic microbiota have been established, the exceeding of which requires correction. In the development of endometritis in cows, the number of *Escherichia coli* and sulfite-producing clostridia in vaginal swabs is at the level of 6.0 ± 0.1 lg CFU/cm^3, staphylococci — 5.9 ± 0.1 lg CFU/cm^3, saprophytic microorganisms of the genus *Bacillus* — 6.8 ± 0.1 lg CFU/cm^3, yeast-like fungi of the genus *Candida* — 5.9 ± 0.1 lg CFU/cm^3.

4. Calves born from cows with dysbiotic disorders of vaginal secretion in 86.1% of cases had dysbiotic disorders of the gastrointestinal tract, namely the number of lactobacilli was at the level of 4.8 ± 0.1 lg CFU/cm^3, bifidobacteria — 4.7 ± 0.1 lg CFU/cm^3. *Escherichia coli* — 6.2 ± 0.1 lg CFU/cm^3, saprophytic microorganisms of the genus Bacillus — 6.0 ± 0.1 lg CFU/cm^3, which in 48.4% of cases led to the development of diarrhea of varying severity.

5. Thus, by studying the qualitative and quantitative composition of the microbiota of vaginal secretions in cows before calving, it is possible to reasonably predict the development of gastrointestinal diseases in calves and endometritis in cows, which makes it possible to develop and take appropriate measures in advance to prevent, treat and prevent recurrence of disease.

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INSUFFICIENTLY STUDIED MINOR VIRAL INFECTIONS IN LIVESTOCK OF UKRAINE

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Summary. For the first time in Ukraine the presence of genetic material of bovine immunodeficiency virus and bovine foamy virus in cattle on Ukrainian farms was detected by scientists of the Laboratory of Leukemia Study and the Laboratory of Molecular Diagnostics of the National Scientific Center 'Institute of Experimental and Clinical Veterinary Medicine'. The associative nature of animal infection with leukemia, immunodeficiency and spumavirus pathogens is recorded. In the future, it is planned to study the properties of pathogens, adaptation to homologous cell cultures and accumulation of viral material in order to develop domestic means of serological diagnosis of immunodeficiency and spumavirus infection

Keywords: epizootic situation, leukemia, immunodeficiency, spumavirus, PCR

Introduction. Among the minor viral diseases of cattle, leukemia, bovine immunodeficiency and bovine spumavirus infection are the most notable. What these diseases have in common is that the pathogens, BLV (Bovine Leukemia Virus), BIV (Bovine Immunodeficiency Virus) and BFV (Bovine Foamy Virus), cause an immunosuppressive state in the body of infected animals, especially during the clinical course of the disease, which makes it impossible to obtain reliable efficacy of treatment and prevention measures, and causes a decrease in the volume and quality of livestock products, loss of valuable gene pool and often death of animals. In addition, pathogens belonging to the retrovirus family also pose a potentially dangerous medical and social threat, as they are structurally similar to the causative agents of AIDS and human T-cell leukemia.

Bovine leukemia, one of the most common slow infectious diseases of cattle, has been well studied in terms of its prevalence in the world livestock industry through the introduction of serological and molecular-genetic research methods. With the introduction of legislation in most European countries, the disease has been eradicated, although there are still some regions with a limited number of infected animals without clinical manifestations of the disease. There is still a significant presence of leukemia in the livestock industry in Canada and the United States—in both North American countries, bovine leukemia occurs not only at the level of seroconversion, but also at the level of clinical manifestation of the disease.

Animals infected with bovine immunodeficiency virus are recorded in many countries of the world, and often associated infection with both infectious immunodeficiency virus and bovine leukemia is recorded. It is worth noting that the serological testing of cattle for immunodeficiency in different countries, based on the materials of individual scientific publications, revealed a significant prevalence of the disease.

Thus, seropositivity rates differ across countries. In the United States, the seropositivity rate is at 4%, whereas in the Netherlands, it is at 1.4%, in Canada, 5.5%, in Germany, 6.6%, and in France, 4%. Immunodeficiency has been determined in several countries, including the UK, Sweden, Costa Rica, Venezuela, New Zealand, and Australia, based on laboratory test results. The rate between seropositive and healthy cattle typically range from 1–7%, although in some herds with a chronic course of the disease (epizootic stationarity), the infection rate can reach as high as 50%. Of the 64% of animals with lymphosarcoma, lymphadenopathy, and other disorders, 74% were infected with the immunodeficiency pathogen (Kolotvin, 2007; Krasnikova, 2011; Supotnitskiy, 2009; Meas et al., 2002).

According to individual authors' materials, infectious bovine immunodeficiency has been reported in Japan, France, Canada, Iran, Argentina, Germany, the Netherlands, Italy, Brazil, Turkey, Cambodia, Pakistan, and Australia with infection rates ranging from 1 to 50% or more (Meas et al., 2002; Romen et al., 2007; Murray et al., 2006).

The scientific literature indicates that BFV seropositivity among cattle in certain livestock farms in developed countries ranges from 30% to 45%, and the resulting infection is prevalent worldwide. Some instances report the occurrence of a two- or even three-variant course, signifying persistent leukemia pathogens, immunodeficiency, and spumavirus infection. The diseases cause significant harm to livestock production, impacting both animal resistance and the volume and quality of products (Romen et al., 2007; Murray et al., 2006; Orr, O'Reilly and Scholl, 2003).

The information reports in the world scientific literature necessitated conducting scientific research on

the epizootic condition of livestock in Ukraine with regards to minor infections. The Laboratory of Leukemia Study of the NSC 'IECVM' initiated such studies within Ukraine.

The purpose of this research is to examine the incidence of minor viral infections in cattle, namely leukemia, immunodeficiency, and spumavirus infection, within the Ukrainian livestock industry.

Materials and methods. We analyzed 10–15 stabilized blood samples obtained from cows in individual farms located in northern and central regions of Ukraine. On these farms, anti-leukemia health improvement measures were taken during the active phase (farms No. 6 in Kharkiv, No. 1 in Kirovohrad, No. 2 in Poltava, and No. 4 in Cherkasy regions) and at the final stages of recovery.

Animals infected with leukemia virus were detected through serological methods, including RID and ELISA, as well as molecular-genetic methods such as PCR. The serological examination of bovine serum for leukemia was carried out on the basis of the Laboratory of Leukemia Study, using the 'Kit of dry components for the serological diagnosis of bovine leukemia in the immunodiffusion reaction' (RID) (manufactured by LLC 'SRE Veterinary Medicine', Kharkiv) and the 'Kit for the detection of antibodies to bovine leukemia virus by ELISA' (manufactured by VMRD, USA).

The total DNA extraction from the biological material samples was performed through the use of the sorbent method (Boom et al., 1990).

The PCR assay was performed using the Thermo Scientific DreamTaq Green PCR Master Mix (2X) reagent kit according to the manufacturer's instructions.

Detection of BFV proviral DNA was performed by standard PCR using primers Int 3 (forward primer, 5`-TCCCGCCTAAAGCTGATAGA-3`) and Int 4 (reverse primer, 5`-CAAACCTGAAATGGCTTGGT-3`), the target of which was the 241-base pair (bp) region of the *pol* gene of the virus (Materniak et al., 2013); detection of BIV proviral DNA was performed using Pol + (forward primer, 5`-GATTTTAGGGAATTAAATAA-3`) and Pol – (reverse primer, 5`-ACCCATCCTTGTGGTAGAACT-3`) primers, the target of which was the 235-bp region of the *pol* gene of the virus (Moody et al., 2002); detection of BIV proviral DNA was performed using BLV-env-3 (forward primer, 5`-CCACAAGGGCGGCGCCGGTTT-3`) and BLV-env-4 (reverse primer, 5`-GCGAGGCCGGTC CAGAGCTGG-3`) primers, the target of which was the 444-bp region of the *env* gene of the virus (Fechner et al., 1996).

The amplification results were visualized through horizontal electrophoresis on a 1.5% agarose gel utilizing 10 μL of the amplification product. Electrophoresis was carried out for 40 min at an electric field intensity of 12 V/cm. The molecular weight marker with a resolution of 100 bp (GeneRuler 100 bp DNA Ladder, Thermo Scientific) was used to determine the amplicon length. The results of the amplification procedure were recorded utilizing the Image Lab 5.2.1 gel imaging software and a BioRad Universal Hood II transilluminator.

Ninety blood samples were collected from cows at seven milk-producing farms in Ukraine's five different regions that were recovering from bovine leukemia. The samples underwent molecular genetic testing to detect genetic material of BIV, BFV, and BLV.

Results and discussion. An epizootic survey was conducted on several livestock farms in central and eastern Ukraine, with implementation of anti-leukemia health measures in conjunction with the Laboratory of Leukemia Study of the NSC 'IECVM'. Specifically, two farms in the central region — Kirovohrad (No. 1) and Poltava (No. 2) — underwent examination of their cattle.

In the first case, the epidemiological survey was based on detecting individual cows infected with leukemia virus in a herd that was previously leukemia-free. In the second case, a high prevalence of leukemia virus infection was found in the herd. In addition to serological tests for leukemia in animals over 6 months old, samples of stabilized blood were collected for molecular genetic studies to identify the genetic material of leukemia pathogens, infectious immunodeficiency, and spumavirus infections in cattle. 10 samples were collected from cows of farm No. 1, and 15 samples from animals of the same category were collected from the herd of farm No. 2.

Similar work was undertaken on farms in Sumy (No. 3), Cherkasy (No. 4), and No. 5, No. 6, and No. 7 in Kharkiv region. Each of these farms implements anti-leukemia health measures which have proven largely efficacious throughout the year. Regular serological tests are performed on livestock beginning at six months of age, followed by the removal of seropositive individuals. The level of seropositivity was reduced from 12% to 0.2%. In some cases, the health improvement measures resulted in the stabilization of the epizootic situation. For instance, despite several serological tests throughout the year, the level of infection among cattle at farm No. 6 in Kharkiv Region remained at 7-8% without declining. It is noteworthy that farm No. 4 in Cherkasy Region falls within the central-western zone. The cattle on this farm exhibit a 13% infection rate of leukemia virus among cows and receive anti-leukemia health measures. Here, along with repeated serological screenings for leukemia in cattle starting at 6 months of age and subsequent isolation of leukemia-compromised animals, 15 stabilized blood samples were obtained to conduct a molecular-genetic research program aimed at identifying the genetic material of leukemia, bovine immunodeficiency, and spumavirus infection in cattle.

The qualitative characteristics of these studies on the isolation of BIV, BFV, and BLV DNA are shown in Table 1.

Table 1 — Results of molecular-genetic study of cattle blood samples

Region	Farm	n	Genetic material detected		
			BLV	BFV	BIV
Kirovohrad	No. 1	10	1	2	1
Poltava	No. 2	15	4	1	—
Sumy	No. 3	10	—	1	1
Cherkasy	No. 4	15	2	1	—
Kharkiv	No. 5	15	—	—	1
	No. 6	15	3	2	—
	No. 7	10	—	1	—

Table 1 shows that the leukemia virus was detected in three cases out of 15 samples of citrated blood taken from the herd of farm No. 6 in the Kharkiv Region, which could be attributed to the fact that the samples were taken from animals of a herd positive for bovine leukemia, where the above disease is in an active stage. In other instances, concerning farms in different regions, the isolation of the leukemia pathogen was sporadic, as anti-leukemia health measures were in their final stages.

Regarding the detection of immunodeficiency and spumavirus infections in cattle, it is important to note that two farms, No. 1 and No. 3, had animals with associative infections. In three other cases, animals from farms No. 2, No. 4, and No. 6 were infected with a combination of leukemia and spumavirus pathogens. In one instance, farm No. 1 had animals infected with a combination of leukemia, bovine immunodeficiency, and spumavirus infections. Thus, the persistence of pathogens causing minor cattle infections such as leukemia, bovine immunodeficiency, and spumavirus infection is observed among the animals on seven farms in five regions of Central and Eastern Ukraine.

Conclusions. 1. The retroviruses responsible for slow infections in cattle, which are leukemia, immunodeficiency, and spumavirus (BLV, BIV, BFV) belong to the Retroviridae family. They have a wide distribution in livestock farms globally.

2. Selective studies of blood samples collected from cattle at risk of leukemia farms in the Kharkiv, Sumy, Kirovohrad, Poltava, and Cherkasy regions demonstrated the presence of BIV and BFV genetic material by utilizing molecular-genetic methods. Additionally, some cases reveal that the pathogenesis of animal infection can be attributed to the association of leukemia pathogens, immunodeficiency, and spumavirus infection in cattle.

3. The above is aimed at a detailed study of the epizootic state of livestock in Ukraine regarding minor viral infections of cattle with the aim of identifying potential areas for improvement in the national program for the regulation of animal products and consumer protection.

Prospects for further use of the obtained results. Indication of the genetic material of immunodeficiency and spumavirus pathogens, study of their properties, adaptation to homologous cell cultures, accumulation of viral material for the development of a domestic serological diagnostic tool.

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Part 2. Biotechnology

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COMPARATIVE ANALYSIS OF THE OPEN READING FRAMES PROTEIN GENES OF GENOTYPE 4 HEPATITIS E VIRUS IN SWINE AND WILD BOAR

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Summary. The goal of this study was to determine the molecular diversity of the open reading frames (ORFs) ORF1, ORF2, ORF3 protein genes from full-length genomes of genotype 4 hepatitis E virus (HEV) from pigs and wild boars at protein and gene level. Statistical technique Shannon entropy was used for mutational analysis of ORF1–ORF3 protein genes to identify amino acid substitutions in the HEV-4 sequences isolated from pigs and wild boars that were most susceptible to mutations. Gene selective pressure for genes was estimated using Tajima’s neutrality test. The ORF regions of 11 swine and 11 wild boar genotype 4 HEV isolates with complete genomes from the GenBank database were analyzed comparatively. The total number of polymorphic sites was determined. Nonsynonymous (amino acid changing) and synonymous (amino acid preserving) substitutions were identified in ORF1, ORF2, ORF3 in swine and wild boar HEV-4 isolates. No evidence of recombination was found for ORFs in 11 swine HEV-4 isolates, ORF2, ORF3 in 8 wild boar HEV-4 isolates. However, a recombination fragment with a length of 430 nucleotides was detected in the ORF1 gene of 3 wild boar HEV-4 isolates. Positive D Tajima factors were determined for ORF1, ORF2, ORF3 genes of swine HEV-4 and ORF1, ORF2 genes of wild boar HEV-4. While a negative value of D Tajima’s factor was determined for ORF3 gene of wild boar HEV-4. Molecular characteristics showing principal distinctions between the open-reading frames of swine and wild boar genotype 4 hepatitis E virus were obtained. Wild boar ORF1 is characterized by lower nucleotide diversity π value (0.144) and higher number of segregated sites S value (1,688) comparing with higher π value (0.159) and lower S value (1,602) of swine ORF1. Positive values of D Tajima’s factor for ORF1, ORF2 ORF3 genes of swine HEV-4 and ORF1, ORF2 genes of wild boar HEV-4 show on positive selection of these genes. Negative value of D Tajima’s factor for ORF3 gene of wild boar HEV-4 indicates onto purifying selection decreasing variability in ORF3 gene of wild boar HEV-4. The largest number of amino acid variation sites (19.2%) was found for wild boar HEV-4 ORF3 followed by swine HEV-4 ORF3 (15.7%) comparing with other swine and wild boars HEV-4 ORFs

Keywords: mutational analysis, entropy analysis, Tajima’s neutrality test, positive selection, purifying selection

Introduction. Hepatitis E virus (HEV) is the causative agent of acute hepatitis — a dangerous liver disease with a mortality rate of about 1%. It is a major public health problem worldwide (Iaconelli et al., 2020). In contrast to known hepatitis viruses various animals are reservoirs for HEV (Pavio, Meng and Renou, 2010; Boadella, 2015).

Genotype 3 and genotype 4 HEV occupy a special place among the eight known genotypes (Wang and Meng, 2021) because of their zoonotic potential. Swine populations and wild boars are the main natural HEV reservoirs with the possibility of cross-species transmission (Fredriksson-Ahomaa, 2019; Salines, Andraud and Rose, 2017). The codon adaptation index for HEV3-4 exceeds 0.5 indicating a high adaptive potential to the host organism (Sun et al., 2020; Bouquet, Cherel, and Pavio, 2012). HEV3–4 is thought to be transmitted by ingestion of food from infected animals (Nan et al., 2017; Grierson et al., 2019).

HEV is a member of the family Hepeviridae, genus *Orthohepevirus*, species *Orthohepevirus A*. The HEV genome is represented by a single-stranded RNA molecule with a length of 7.2 knt consisting of three open reading frames (ORF). ORF1 has the most length of them, encodes non-structural proteins involved in HEV replication, and contains several functional domains (Ahmad, Holla and Jameel, 2011). ORF2 encodes the capsid protein which is a main structural component of the virion. The ORF2 protein is the main immunogenic target of neutralizing antibodies and exists in two forms — $ORF2^{S}$ (secreted form) and $ORF2^{C}$ (capsid associated form). In this case, translation is initiated from two different codons located at a distance of 15 amino acids (Yin et al., 2018). ORF3 encodes a multifunctional small phosphoprotein involved in HEV replication and pathogenesis (Kenney and Meng, 2019).

HEV as RNA virus exists as a mixture of quasispecies, i. e. closely related variants (Lauring and Andino, 2010). In spite of the similar transmission and the ability to cause chronic hepatitis, HEV-3 and HEV-4 differ in clinical manifestation and pathogenesis. In particular, humans and animals infected by HEV-4 show

significantly higher levels of alanine aminotransferase which is a marker of liver damage. HEV-4 causes fulminant hepatitis and early cirrhosis more often comparing with HEV-3 (Takahashi and Okamoto, 2014; Ohnishi et al., 2006; Perumpail et al., 2015).

The mutations and recombinations are the main mechanisms genomic diversity of viruses that can change their biological (contagiousness, virulence, etc.) or phenotypic properties (Domingo and Holland, 1997). Mutation process along with natural selection is a key factor of the evolution. HEV strains are characterized by the significant level of the genomic diversity despite the only one serotype existence (Okamoto, 2007).

The most HEV encoding regions are under the purifying (or negative) selection directed against arising mutations and changing amino acid sequences (Smith D. et al., 2012). The regions of HEV genome with a high level of amino acid substitutions are localized at the ORF2 N-end and ORF3 C-end. These regions are under the positive selection directing onto mutations spreading and fixation (Chen et al., 2012).

A region of overlapping reading frames for HEV3–4 is under the positive selection (Brayne et al., 2017). Among four main HEV genotypes (HEV1–4) HEV3–4 are the most diversified that may explain broad host range (Lara, Purdy and Khudyakov, 2014). Genome variability of viruses depends on its copying accuracy degree. The transcription is a source of HEV high mutation rate and genomic diversity (Van et al., 2016). HEV mutation rate of clinical isolates was found to be 1.5 nucleotide substitution per site in a year (Takahashi and Okamoto, 2014).

The ratio of the related rate of nonsynonymous mutations (dn) tothe related rate of synonymous mutations (ds) d*n*/d*s* is used to assess the variability deviation of virus genomes from the model of neutral molecular evolution (Kimura, 1991) and to determine selection mode. A ratio d*n*/d*s* is used by many researches for estimation of selection direction, selection strength for protein coding sequences and useful to distinguish various processes of evolution (Aziz et al., 2022; Gutierrez, Escalera-Zamudio and Pybus, 2019; Dasmeh et al., 2014). The ratio of numbers of synonymous substitutions per synonymous site / nonsynonymous substitutions per nonsynonymous site is used as a marker of the negative (< 1) or positive (> 1) selection.

This ratio was used for the characteristics of HEV quasispecies diversity at the acute phase of hepatitis E in solid-organ transpant patients. M and P capsid domains of HEV quasispecies in patients who developed chronic infection was found to be under the negative selection (Lhomme et al., 2012).

Statistical tests can be based on the estimation of difference between the number of single substitutions and the total number of substitutions (Fu and Li, 1993) or average value of pairwise nucleotide differences between sequences (Fu, 1997). A widely used efficient Tajima's test takes into account the number of variable sites and the average differences for large data sets in determining selection type (Tajima, 1989; Mohamed et al., 2019; Niczyporuk et al., 2020; Jadhav et al., 2020). The Tajima's test is based on the estimate of genetic diversity θ (substitutions per site) of a sequence alignment. Negative value of Tajima's indicates purifying selection, the value greater than zero indicates the positive selection (Yang, and Bielawski, 2000).

In the biological systems the processes of metabolism, energy and information exchange can be accompanied by both an increase and a decrease in entropy. A Shannon entropy is used for structural analysis of the biological systems condition at the macromolecules level and for determination of the degree of genetic variability at each amino acid or nucleotide position (Shannon, 1997). A higher Shannon entropy value at a sequence position indicates more variability in that position. A Shannon entropy value of zero indicates an invariant column of nucleotides / amino acid residues for all variants. Shannon entropy values were used for investigation of the different regions variability of genotypes 1, 3 and 4 HEV. The entropy values in X domain, RNA-dependent RNA polymerase domain, ORF2, ORF3 were determined to be the highest in HEV-3 and HEV-4 comparing with HEV-1 (Muñoz-Chimeno et al., 2022).

In this paper molecular characterization of three open reading frames in swine and wild boar HEV-4 on the macromolecules level was performed by computational methodologies.

Materials and methods. Full-length genomes for isolated from pig and wild boar genotype 4 HEV strains were obtained by searching the NCBI Nucleotide Database using the taxonomic identifier (txid) 1678143, along with associated metadata on host, country, and date of sampling. The parameters of the strains considered for the present study are listed in Table 1.

Table 1 — Parameters of isolated from swine and wild boars HEV-4 strains analyzed in the present study

	Strain/Isolate	GenBank record	Country	Year
Swine HEV-4	HB-S3	KX531115	China	2014
	HN-JY40	KM253769	China	2015
	CHN-SD-sHEV	KF176351	China	2011
	KM01	KJ155502	China	2010
	hb-3	GU361892	China	2008
	CHN-XJ-SW33	GU119960	China	2009
	bjsw1	GU206559	China	2008
	IND-SW-00-01	AY723745	India	2006
	swGX32	EU366559	China	2007
	BeSW67HEV4-2008	OM388298	Belgium	2008
	SS19	JX855794	China	2011

Table 1 — continuation

	Strain/Isolate	GenBank record	Country	Year
Wild boar HEV-4	G4HEV121-12cc	LC657084	Japan	2008
	2003-TL01	LC646471	Japan	2003
	JTF-Yamagu11	AB698654	Japan	2017
	CN-HuN2	MZ544007	China	2020
	wbJGF_08-1	AB602440	Japan	2008
	CN-GS3	MZ544006	China	2020
	CN-XJ7	MZ544005	China	2019
	CN-IM14	MZ544004	China	2019
	CN-JL23	MZ544003	China	2018
	CN-JL14	MZ544002	China	2018
	CN-CQ3	MZ544001	China	2019

The ORF genes sequences for the present study were divided into two datasets. Dataset 1 contained ORF 1 — ORF3 genes sequences from 11 swine HEV-4 strains. Dataset 2 consisted of ORF1–ORF3 genes sequences isolated from 11 HEV-4 strains of wild boar. Alignments for all two datasets were carried out using Molecular Evolutionary Genetics Analysis (MEGA) software (version 6.06) (Tamura et al., 2013). BioEdit (version 7.2.5) (Hall, 2013) software was used for mutational analysis of ORF1–ORF3 protein genes to determine the amino acid substitutions in the HEV-4 sequences isolated from pigs and wild boars. Gene selective pressure for genes was estimated using the Tajima's neutrality test by MEGA 6. Translation of nucleotide sequences in amino acid ones with following codon analysis was performed by BioEdit.

Shannon entropy is a useful quantification of diversity at a single position. Entropy plots for ORF1–ORF3 protein sequences, representing the amount of amino acid (and hence nucleotide) variability through each column in aligned sequences, were calculated by BioEdit.

Aligned ORF1–ORF3 gene sequences were screened for recombination with recombination detection program RDP4 (version 4.101) (Martin et al., 2015), using five available methods (RDP (Martin and Rybicki, 2000), GENECONV (Padidam, Sawyer and Fauquet, 1999), BootScan (Martin et al., 2005), MaxChi (Smith J., 1992), SiScan (Gibbs, Armstrong and Gibbs, 2000)) with default settings.

Results. More than thirty years have passed since the isolation of the first animal HEV strain (in swine), which was reported in 1990 (Reyes et al., 1990; Kordyum, 2001).

Analysis of mutations in ORF1–ORF3 protein genes. ORF1, ORF2, ORF3 gene sequences were restricted from full-length genomes of pig and wild boar genotype 4 HEV isolates. Nonsynonymous (amino acid-changing) and synonymous (amino acid-preserving) substitutions were identified in the ORF1, ORF2, ORF3 proteins of swine and wild boar genotype 4 HEV isolates (Table 2).

Table 2 — The number of polymorphic sites in aligned ORF1–ORF3 proteins of swine and wild boar HEV-4 comparing with complete length (in paranthesis) and position of hypervariavle region (HVR)

	ORF1	ORF2	ORF3
Swine HEV-4	91 (1,708) HVR: 719-789, 1,515-1,708	43 (623) HVR: 624-661	18 (114)
Wild boar HEV-4	78 (1,708) HVR: 717-789	34 (660)	22 (114)

Nonsynonymous substitutions in the ORF3 proteins of swine and wild boar genotype 4 HEV are summarized in Table 3.

Table 3 — The specific codon positions along with nonsynonymous (amino acid-changing) substitutions in the ORF3 protein of swine HEV-4 (left) and wild boar HEV-4 (right). The sequences GU361892 and LC646471 were used as a reference genotype for swine HEV-4 ORF3 and wild boar HEV-4 ORF3, respectively. Unique substitutions for ORF-3 of swine and wild boar HEV-4 are highlighted

Codon position	GenBank record	Amino acid residues substitution
Swine HEV-4		
2	EU366959, GU119960, GU206559	E (A)
32	AY723745	A (T)
34	EU366959, GU119960	A (V)
35	EU366959, GU119960	A (T)
39	KM253769	H (P)
68	AY723745	Q (R)
71	KF176351	Q (R)
73	GU119960, EU366959	P (Q)
74	AY723745, JX855794, OM388298	P (Q)
82	EU366959, GU119960, KX531115	G (D)
83	EU366959, GU119960	R (N)
	GU206559, AY723745, JX855794, KJ155502, KM253769, OM388298	R (S)
84	GU119960, KJ155502, KM253769	Q (R)
86	EU366959, GU206559	A (V)
89	JX855794	A (V)
93	AY723745, EU366959, JX855794	V (A)
94	AY723745	T (I)
102	GU119960	P (L)
104	KF176351, KJ155502, KM253769	V (A

Table 3 — continuation

Codon position	GenBank record	Amino acid residues substitution
Wild boar HEV-4		
34	LC657084, MZ544003	A (V)
39	AB698654, MZ544004	A (T)
45	LC657084	V (A)
67	AB698654, MZ544001	L (S)
69	AB698654, MZ544001, MZ544003 MZ544004, MZ544005, MZ544006, MZ544007	P (L)
72	MZ544006	Q (R)
73	MZ544002	P (L)
74	MZ544001, MZ544002, MZ544004, MZ544005, MZ544006, MZ544007	Q (P)
82	MZ544001	G (D)
83	MZ544001, MZ544007	S (R)
	MZ544006	S (P)
84	MZ544003, MZ544005	Q (R)
85	MZ544005	P (S)
86	AB698654, MZ544001, MZ544002, MZ544004, MZ544005, MZ544006, MZ544007	V (A)
88	AB698654	S (L)
89	MZ544003, MZ544005	A (V)
91	AB698654, MZ544001, MZ544002, MZ544003, MZ544004, MZ544005, MZ544006, MZ544007	P (L)
93	AB698654, MZ544002, MZ544003, MZ544007	V (A)
94	AB698654	T (I)
95	AB698654, MZ544001, MZ544002, MZ544003, MZ544004, MZ544006	N (S)
	MZ544007, LC657084	N (K)
100	MZ544003	P (L)
101	AB698654	L (P)
104	MZ544001, MZ544002, MZ544004, MZ544005, MZ544006, MZ544007	V (A)

Sequences were obtained from GenBank records and annotated by year of sampling. Data for the nonsynonymous substitutions in the ORF1 and ORF2 of swine and wild boar HEV-4 isolates are not shown.

Five unique substitutions were identified for swine ORF3 and nine ones for wild boar ORF3 in several HEV-4 isolates. These substitutions result in changing ORFs surface properties. For example, Q (P) amino acid residue substitution leads in decreasing polarity of wild boar HEV-4 ORF3 protein for 6 HEV isolates (position 74, Table 3) because P amino acid residue is characterized smaller polarity comparing with that of Q amino acid residue. N (S) and N (K) amino acid residues substitution results in increasing polarity of wild boar HEV-4 ORF3 protein for 8 HEV isolates (position 95, Table 3) because K and S amino acid residues are more polar comparing with that of N amino acid residue.

No evidence of recombination was found in the alignment for ORF1 gene sequence of pig isolates, ORF2, ORF3 gene sequences of pig and wild boar genotype 4 HEV isolates. But recombination was determined for ORF1 gene of wild boar isolates. Recombination fragment with length of 430 nucleotides (nt) was identified for three isolates from set of 11 ORF1 gene HEV-4 isolates. Fig. 1 graphically illustrates statistical evidence of recombination events between potential recombinant AB698654 (Japan, 2017), potential major parent MZ544005 (China, 2019), potential minor parent MZ544006 (China, 2020).

Analysis of Shannon entropy in ORF1–ORF3 protein sequences. Entropy measures the variability within site and assigns high score to highly variable sites and a lower score to less variable sites. Shannon entropy is a measure of the lack of information content (how could predict the position for a new incoming sequence) at each position in the alignment. By other words, entropy is a measure of the lack of predictability for an alignment position. For example, if any nucleotide (A, T, G or C) from four ones can be at position X with a frequency of 0.25, then information content has been reduced to 0, and the entropy is at maximum variability.

And, contrary, if there are N sequences in an alignment and at position K there is only one type nucleotide (for example, T) in all sequences, it can be assumed that there is a maximum information for position K. Assumption about nucleotide G at position K of another homologous sequence would be correct. That means a maximum information for position K, and the entropy in that case is 0.

A total of 92, 34, and 22 amino acid variation sites were identified by entropy analysis in dataset I for ORF1, ORF2 and ORF3 proteins of swine HEV-4. Variability is calculated as the entropy for each amino acid residue position. Entropy percentages for swine ORFs are as follow: ORF1 — 5.3% (91/1,708), ORF2 — 6.9% (43/623), and ORF3 — 15.7% (18/114).

A total of 78, 34, and 22 amino acid variation sites were identified by entropy analysis in datasets II for ORF1, ORF2 and ORF3 proteins of wild boar HEV-4. Entropy percentages for wild boar ORFs are as follow: ORF1 — 4.6% (78/1,708), ORF2 — 5.2% (34/660), and ORF3 — 19.2% (22/114). Entropy analysis revealed that wild boar ORF3 observed the largest variation (Fig. 2A) followed by swine ORF3 (Fig. 2B).

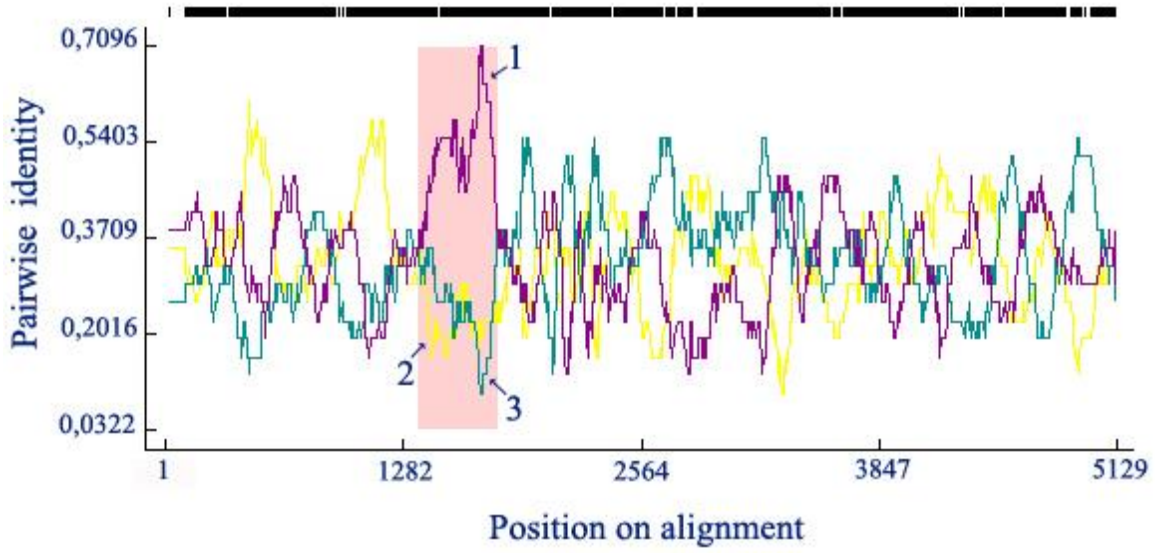

Figure 1. Plot of recombination events for ORF1gene of aligned three wild boar genotype 4 HEV isolates from set of 11 sequences. Different coloured lines indicate different sequence pairs: 1 — recombinant AB698654–minor parent MZ544006; 2 —major parent MZ544005–minor parent MZ544006; 3 — recombinant AB698654–major parent MZ544005. Pink rectangular area indicates ORF1 fragment with recombination length of 430 nucleotides of high identity between potential recombinant and closely related sequences.

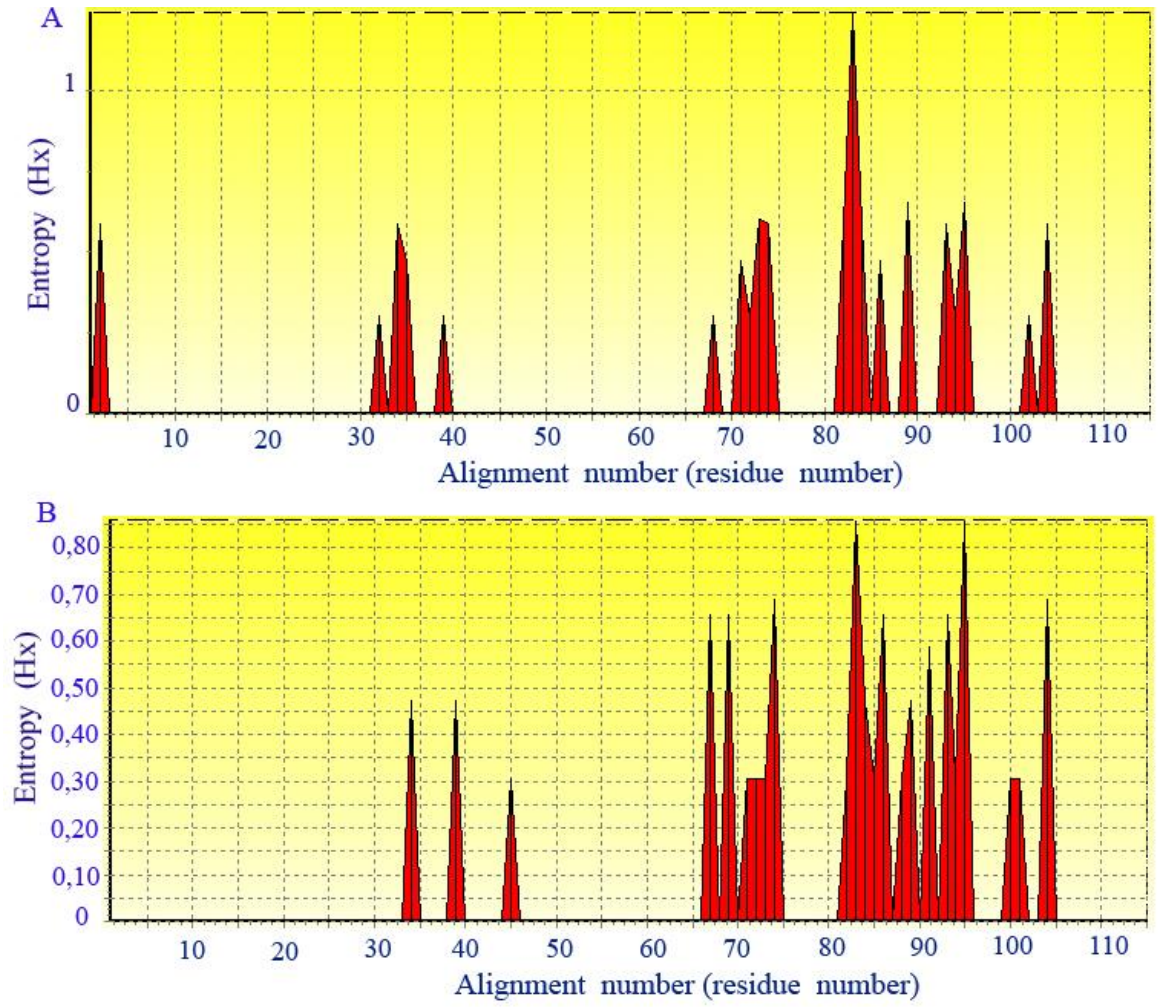

Figure 2. Entropy plots as a measure of diversity at each amino acid position for aligned amino acid sequences in ORF3 of swine HEV-4 (A) and wild boar HEV-4 (B).

Analysis of positive and purifying selection in ORF1–ORF3 protein genes. To identify ORF regions under positive or purifying selection, we estimated D value using Tajima's neutrality test. Parameter D indicates rating of correspondence nucleotide substitutions type to neutrality hypothesis for analyzed sequences. Deviation of observed diversity from model of neutral evolution may be determined by Tajima's test.

Veracious positive D factor values may indicate on sharp decreasing virus population or compensatory selection. ORF1, ORF2, ORF3 genes of swine HEV-4 and ORF1, ORF2 genes of wild boar HEV-4 consisted of genes under positive selection as indicated by positive D factors in Table 4, respectively. The selection pressure revealed the prevalence of positively selected sites in mentioned genes.

Table 4 — Parameters of mutational analysis of ORF1–ORF3 protein genes of HEV genotype 4 from pig and wild boar. Parameter D is the Tajima test statistic which indicates rating of correspondence nucleotide substitutions type to neutrality hypothesis for studied sequences

		m	S	p_s	Θ	π	D
Swine HEV-4	ORF1	11	1,602	0.361	0.123	0.159	1.409
	ORF2	11	558	0.281	0.096	0.119	1.163
	ORF3	11	43	0.124	0.042	0.045	0.340
Wild boar HEV-4	ORF1	11	1,688	0.330	0.112	0.144	1.360
	ORF2	11	512	0.258	0.088	0.109	1.193
	ORF3	11	48	0.139	0.048	0.046	–0.143
	ORF3	10	48	0.136	0.048	0.046	–0.278

Abbreviations: m — number of sequences, n — total number of sites, S — number of segregating sites (number of polymorphic sites in sequences), p_s — S/n, Θ — p_s/a_1, π — average nucleotide diversity.

However, ORF3 gene of wild boar HEV-4 was found to be under purifying selection as indicated by negative D factor, i. e. –0.143. Negative value of D Tajima's factor indicates onto recent population growth or purifying selection decreasing variability.

This suggests that the ORF1, ORF2 genes as of swine as of wild boar HEV-4 evolution is mainly driven by positive selection. Positive selection was obtained for ORF3 gene of swine HEV-4 too. While prevalence of purifying selection in ORF3 gene of wild boar HEV-4 was observed.

Estimated number of segregated sites (S) in swine and wild boar ORF2–ORF3 is in accordance with nucleotide diversity (π). The highest S value as for swine ORF2–ORF3 as wild boar ORF2–ORF3 is correlated with highest π value for swine and wild boar ORFs HEV, respectively. But quit different situation is for comparing S and π values in swine and wild boar ORF1. Wild boar ORF1 is characterized by lower π value (0.144) and higher S value (1,688) in comparison with higher π value (0.159) and lower S value (1,602) of swine ORF1.

Conclusions. New significant comparative information on the ORF1–ORF3 proteins of swine and wild boar genotype 4 HEV was obtained.

Positive values of D Tajima's factor for ORF1, ORF2, ORF3 genes of swine HEV-4 and ORF1, ORF2 genes of wild boar HEV-4 show on positive selection of these genes.

Negative value of D Tajima's factor for ORF3 gene of wild boar HEV-4 indicates onto purifying selection decreasing variability in ORF3 gene of wild boar HEV-4.

Genomic diversity in the ORFs protein genes of swine and wild boar HEV-4 isolates is quite different. For swine and wild boar ORF1 and ORF2 lower nucleotide diversity π corresponds to lower number of segregated sites S value. But wild boar ORF1 is characterized by lower nucleotide diversity π value (0.144) and higher number of segregated sites S value (1,688) comparing with higher π value (0.159) and lower S value (1,602) of swine ORF1, respectively.

Wild boar HEV-4 ORF3 observed the largest number of amino acid variation sites (19.2%) followed by swine HEV-4 ORF3 (15.7%) comparing with other swine and wild boars HEV-4 ORFs.

Further computational approaches are required to compare obtained results with swine and wild boar genotype 3 HEV.

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BIOLOGICAL PROPERTIES OF NANOMATERIALS (LITERATURE REVIEW)

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Summary. In the article reviewed and discussed literature data on biological properties of nanomaterials. The biosafety of nanomaterials is a complex and multifaceted issue that demands a comprehensive, science-based approach. Modern environmental and economic factors should be considered in this regard. The EU's nanotechnology policy is based on 'an integrated, safe and responsible approach' (Communication from the Commission to the European Parliament, the Council and the European Economic and Social Committee. Regulatory aspects of nanomaterials. SEC(2008) 2036 / COM(2008) 366 final). Based on the findings of toxicity and antimicrobial activity studies, metal nanoparticles appear to be a favorable choice as antibacterial agents in developing new disinfectants. However, further measures must be taken to ensure the safe and environmentally friendly use of metal nanoparticles (MeNPs). To achieve this, it is crucial to establish toxicity parameters for MeNPs of various compositions, sizes, and concentrations. These parameters must be compared and evaluated alongside the potential effects of MeNPs on laboratory and target animals (*in vivo*), as well as their antibacterial performance against microorganisms of different strains (*in vitro*). Thus, the investigation of possible hazards associated with the use of metal nanoparticles can be effectively achieved by analyzing the fundamental systemic characteristics of biological systems under both *in vivo* and *in vitro* conditions, taking into account various aspects such as physiological, biochemical, immunological, genetic and cytological responses that may be affected by toxic effects. The literary sources analysis and article publication were conducted under the National Research Foundation of Ukraine project No. 2021.01/0076 'Development of a novel, nanoparticle-based disinfectant for deactivation of pathogens causing emergent infectious diseases'

Keywords: metal nanoparticles, cytotoxicity, antimicrobial activity

Introduction. The control of bacterial bacterial diseases in agriculture and livestock requires the development of new antimicrobial drugs and/or disinfectants to prevent infectious diseases in animals and increase their overall productivity (Landers et al., 2012; Manyi-Loh et al., 2018; Paliy et al., 2020). Several approaches have been proposed to overcome or at least reduce the pressure of bacterial infections caused by resistant strains, including antimicrobial peptides, bacteriophages, targeting quorum sensing and biofilm formation, etc. (Lei et al., 2019; Romero-Calle et al., 2019; Piewngam et al., 2020; Rodionova et al., 2021). All of the above strategies have their advantages and disadvantages. However, despite the obvious progress in the development of biocidal prophylactic and therapeutic agents as alternatives to antibiotics, and even promising results of clinical trials, their implementation in clinical medicine is still of limited importance.

The aim of the study was to review and to discuss literature data on biological properties of nanomaterials.

Results and discussion. Nanoparticles (NPs), materials with sizes ranging from 1–100 nm, are particularly effective in destroying microorganisms. Metal nanoparticles (MeNPs) have gained attention as potential biocides due to their unique features, making them promising candidates for high antimicrobial capacity. Their small size and distinctive properties enable nanomaterials to penetrate prokaryotic cells, allowing for toxicity. For instance, the large surface-to-volume ratio of NPs increases their interaction area with bacteria, and they can be functionalized with ligands that facilitate contact with microorganisms. However, accumulating evidence suggests that MeNPs exhibit considerable potential as antimicrobial agents. They can either amplify the effects of antibiotics or provide bactericidal effects on their own (Gao and Zhang, 2021). It is worth noting that bacteria have a limited capacity to develop resistance to nanomaterials because of the diverse mechanisms of their antibacterial activity. These include the formation of reactive oxygen species (ROS), release of metal ions, damage to bacterial membranes and cell walls, and intracellular macromolecules like proteins and DNA (Niño-Martínez et al., 2019).

In most cases, the toxicity of MeNPs is attributed to the metal ions released; the antimicrobial activity is highly dependent on their physicochemical properties, such as surface, size, and charge. Furthermore, these features can be engineered to maximize the contacts between microorganisms and MeNPs, biofilm penetration, and antimicrobial efficacy of the latter. The size of MeNPs is a significant factor, as it determines whether nanoparticles penetrate microbial cells and biofilms, thus increasing their toxicity (Amaro et al., 2021). Considering the mechanisms of antimicrobial

activity typical of MeNPs (induction of oxidative stress, release of metal ions, DNA damage, ATP depletion, non-oxidative pathways such as changes at the transcriptional and proteomic levels), bacteria may acquire resistance to such agents to a lesser extent compared to conventional antibiotics (Slavin et al., 2017; Lee et al., 2019). The use of disinfectant nanopreparations is one of the strategies to combat antibiotic resistance. However, MeNPs have been shown to be toxic to eukaryotic cells (Vimbela et al., 2017). Because of this fact, it is crucial to develop nanoparticles that are selectively toxic to prokaryotic cells while maintaining a dose-response compromise between efficacy and toxicity.

A growing number of publications indicate that bacteria develop certain mechanisms to counteract silver nanoparticles, including the production of flagellin, which causes agglomeration of MeNPs, the synthesis of pigment to bind them, or the formation of efflux pumps to remove nanoparticles (Panácek et al., 2018; Niño-Martínez et al., 2019; McNeilly et al., 2021). This indicates the relevance of developing alternatives to silver (gold, silver, copper, their oxides, etc.) that have a strong bactericidal effect (Wang et al., 2017; Sánchez-Lopez et al., 2020). Some studies have shown that cerium oxide (CeO_2) NPs have antimicrobial effects (Farias et al., 2018; Pop et al., 2020). The antimicrobial activity, the effect of medium-sized (1–2 nm) and (10–12 nm) CeO_2 NPs on DNA cleavage, microbial cell viability, and biofilm formation inhibition have been proven, and their low cytotoxicity to eukaryotic cells has been demonstrated (Yefimova et al., 2023). Thus, CeO_2 NPs demonstrate DNA cleavage activity when using plasmid DNA as a target DNA molecule, significantly inhibit the viability of microbial cells against *E. coli*; the maximum biofilm inhibition ability of 61.06% for *P. aeruginosa* and 83.86 % for *S. aureus* was achieved using smaller CeO_2 nanoparticles (1–2 nm) at a concentration of 500 mg/L.

Vanadate compounds have been shown to exhibit antiradical and antioxidant properties (Francik et al., 2011). At the same time, there are reports on the toxicity of some vanadium oxides and salts and their pro-oxidant effects, which indicates that vanadate compounds are unsuitable for use in the pharmaceutical industry due to significant side effects (Hosseini et al., 2013). Nevertheless, recent studies have shown that the biological effects of vanadium-containing nanoparticles can be completely different from those of vanadium compounds. For example, V_2O_3 nanowires have been shown to have strong antioxidant properties (enzyme-mimetic properties) in contrast to the prooxidant properties of V_2O_3 NPs (Vernekar et al., 2014; Ghosh et al., 2018). In this context, these compounds are an example of multifunctional nanomaterials with variable redox activity. VO_4:Eu_3+ NPs exhibit strong ROS scavenging ability against X-ray-induced ROS, anticancer activity, and anti-inflammatory properties (Bishayee et al., 2000; Harati and Ani, 2006; Maksimchuk et al., 2020). In addition, it is worth noting that these nanomaterials have low toxicity against eukaryotic cells. However, it is known that the antimicrobial effects of metal-containing nanoparticles depend on their shape and size (Dong et al., 2019). Larger CeO_2 NPs (10–12 nm) have been shown to be less toxic to eukaryotic cells (red blood cells) compared to smaller ones (1–2 nm) (Yefimova et al., 2023).

The cytotoxicity in a fibroblast cell model and the antimicrobial activity of $LaVO_4$:Eu_3+ NPs and $GdVO_4$:Eu_3+ NPs were analyzed (Gonca et al., 2022). The effect of the nanomaterials was evaluated using MTT assay, neutral red absorbance, and scratch assays. It turned out that $GdVO_4$:Eu_3+ NPs are less toxic to eukaryotic cells compared to $LaVO_4$:Eu_3+ NPs. Both types of nanoparticles exhibited antimicrobial activity, and the highest MIC values were shown by NPs $GdVO_4$:Eu_3+ and amounted to 64 mg/L for *E. hirae, E. faecalis* and *S. aureus*, respectively. However, GdYVO$_4$:Eu3þ NPs promoted depolarization of mitochondrial membrane (DWM) of host immune cells and leukocyte apoptosis at high concentrations (Gonca et al., 2022).

In vitro studies have shown that in determining the cytotoxic effect of nanomaterials, dose-dependent prooxidant effects have been identified (Meng et al., 2007; Jia et al., 2009; Li et al., 2009; Colon et al., 2009).

The investigation of the cytotoxicity of 71 nm NPOZn on human bronchoalveolar carcinoma cell culture showed a dose-dependent decrease in cell viability at a concentration of 10–14 μg/mL for 24 hours (Sahoo et al., 2007). Increased levels of malondialdehyde (MDA) and lactate dehydrogenase (LDH) activity were observed, indicating signs of oxidative stress and cytolysis, respectively. Furthermore, DNA damage was visible in the gel electrophoresis conducted on isolated cells. Similar results were obtained by the authors when exposing human bronchoalveolar carcinoma cells to 15 and 46 nm $NPSiO_2$ in culture.

Cultivation of BRL 3A rat liver cells for 24 hours with the presence of 10 nm and 15 nm NPAg at concentrations of 5–50 μg/mL caused a significant shift in the functional state of mitochondria (Hussain et al., 2005; Lok et al, 2007). In the presence of MeNPs such as $NPFe_3O_4$ (30–47 nm), NPAl (30–103 nm), $NPMgO_3$ (30–150 nm), and $NPTiO_2$ (40 nm) at a concentration of 10–50 μg/ml, cell state was not affected. However, at a concentration of 10–250 μg/ml, these particles contributed to decreased viability and LDHase release in the culture medium. NPAg induced ROM (reactive oxygen metabolites) generation, reduction of glutathione content and mitochondrial membrane potential. The authors suggest that oxidative stress mediates the cytotoxic effects of these MeNPs.

The pronounced cytotoxicity of NPAg was determined in a model of mammalian stem cells —

sperm progenitors and stem cells in hair follicles (Braydich-Stolle et al., 2005). Comparative evaluation of NPAg, NPMo, NPAl and NPSd in the exposure with testicular stem cells of 6-day-old mice for 48 hours revealed higher toxicity of NPAg and NPCd on spermatogenesis in experimental animals (Braydich-Stolle et al., 2005).

The toxic effects of NPAg require special attention of researchers since these NPs have been used in medicine for more than 10 years for bactericidal purposes in bone implants, dressings, and other materials (Alt et al., 2004).

It is believed that the toxicity of Ag itself to mammals is relatively low, but, especially in its ionic water-soluble form, it is toxic to aquaculture (Kim et al., 2007; Scheringer, 2008). It is important that information on the mutagenic and carcinogenic activity of this bimetal compounds is limited.

When studying the cytotoxicity of Cobalt, Nickel, Titanium, and Silicium nanoparticles on human endothelial cells *in vitro*, a dose-dependent decrease in their viability was found to be greater under the influence of NPCo and NPNi, accompanied by the expression of proinflammatory cytokines (interleukin-8, E-selectin, and ICAM-1) (Peters et al., 2004).

Cultivation of cardiovascular endothelial cells in the presence of nanoparticles at a concentration of 0.05 and 0.20 mg/l caused an increase in the expression of mRNA interleukin-4 and ecstaxin, which have an anti-inflammatory effect (Yacobi et al., 2007).

It was found that dust storm nanoparticles of 2.5 nm in size for 2 hours in experiments on isolated alveolar macrophages inhibit the activity of Na, K- and Ca, Mg-ATPases of the plasma membrane, affecting its fluidity, stimulate the release of LDHase from cells, reduce the intracellular content of glutathione and lead to the accumulation of LPO products (Meng et al., 2007).

When carbon nanotubes interacted with rat macrophages and human lung cells for 24 hours at a concentration of 10–100 μg/mL, the nanoparticles entered the cell cytoplasm, resulting in decreased cell viability as recorded by the tetrazolium test (Zhu et al., 2007). Incubating cells with carbon nanotubes led to an intracellular accumulation of ROM and a subsequent reduction in mitochondrial membrane potential. The presence of DNA damage induced by nanotubes in mouse embryonic stem cells necessitates a principled approach to utilizing such nanomaterials in biotechnology.

Studies have shown that single-walled nanotubes possess more toxicity than multi-walled tubes and fullerenes (Donaldson et al., 2006). Additionally, their cytotoxicity is high, even at lower concentrations of 0.38 μg/ml, leading to the disruption of cell morphology as well as mitochondrial and phagocytic functions.

The formation of oxidative stress and the accumulation of toxic lipid peroxidation (LPO) products are commonly associated with the mechanisms of toxic effects of carbon and other nanoparticles, as reported by most authors (Li et al., 2003).

The LC_{10} and LC_{50} values of cytotoxicity for the fraction of Fe, Al, Ca, Na, K, Mg, Pb particles in the nanophase present in the cultivation of human lung epithelial cells of the L_{132} line are 18.8 and 75.4 μg/mL, respectively. This fraction induced concentration- and time-dependent alterations in LPO and superoxide dismutase (SOD) activity, the formation of 8-hydroxy-2-deoxyguanine, poly(ADP)-ribosylation, secretion of tumor necrosis factor and NO, as well as activation of inducible NO synthase (Garcon et al., 2006).

Oberdorster et al. (2001, 2005) believe that the mechanisms of cytotoxicity and genotoxicity of carbon nanoparticles occur via pathogenetic pathways: (a) the reactive surface of nanoparticles in interaction with the cell receptor causes oxidation of proteins and lipids of the cell membrane, accumulation of toxic LPO products against the background of antioxidant depletion, which leads to an increase in the intracellular calcium content and gene activation; (b) as a result of oxidative stress, transition-valence metals are released; (c) cell membrane receptors activate transition-valence metals; (d) intracellular transcytosis of nanoparticles in mitochondria induces intracellular oxidative stress and gene expression.

If at low concentrations nanoparticles cause a moderate prooxidant effect against the background of activation of antioxidant system factors: catalase, oxidized and reduced glutathione, SOD and active induction of the antioxidant metallothionein (Oberdörster, 2001; Garcon et al, 2006; Brunner et al., 2006), while high levels of oxidative stress and inhibition of most of the AOS factors, with high levels of metallothionein.

In vivo and *in vitro* experiments have hown that the cytotoxicity of MeNPs and other nanoparticles is caused by genetic and mutagenic effects and the formation of oxidative stress with the formation of ROM in the processes of lipid peroxidation and oxidative modification of proteins (Zhang et al., 2003; Yamakoshi et al., 2003; Jia et al., 2005; Brunner et al., 2006).

When examining the molecular mechanisms behind the adverse impact of stressors on living organisms, it is important to note that the free radical theory of stress has been the most extensively researched in recent times (Pomatto and Davies, 2018; Di Meo and Venditti, 2020; Hitchler and Domann, 2021). The development of stress can be classified into three stages: mobilization (anxiety), resistance, and exhaustion. During the anxiety stage, the body undergoes catabolism, which results in accelerated breakdown of organic substances in tissues, negative nitrogen balance, and increased permeability of blood vessel walls. This phase typically lasts between 4 to 48 hours. If the stress factor is excessively intense, the animal may die.

If the body's defenses fail to overcome the stress, it enters the stage of resistance. During this stage, the metabolism returns to normal, anabolic processes occur, and the white blood cell count, corticosteroid hormone levels, and body weight increase. The resistance stage can last from several hours to several days, and sometimes even weeks. The development of stress ends at the stage of resistance when the stressor stops and the body's metabolism returns to normal. If stressors persist, they can deplete an organism's adaptive capabilities, halt development, and initiate the stage of exhaustion. During this phase, dystrophic changes can occur in organs and tissues while catabolism becomes the dominant metabolic process. Extended periods of exposure to stressors can result in altered metabolism and even animal death (Fan et al., 2002; Donaldson et al., 2003; Dahiya et al., 2007). This indicates the occurrence of destructive processes linked to the denaturation of antioxidant enzymes caused by toxic products of lipoperoxidation, the oxidative alteration of proteins, and other metabolites (Guéraud et al., 2010; Sharifi-Rad et al., 2020; Dimova et al., 2022).

The increase in the intensity of destructive processes in the liver of animals due to the development of oxidative stress under the influence of metal nanoparticles against the background of feed stress is also indicated by the dynamics of enzymes in the blood plasma of rats (Li et al., 2015; Samrot et al., 2022).

As described by Gharbi et al. (2005), after 3 weeks of intraperitoneal administration of an aqueous suspension of fullerenes C_{60} at a concentration of 0.5–2.0 g/kg body weight, no acute or subacute toxicity was detected in the histopathological data of the parenchymal structure of the liver without signs of inflammation or fibrosis. At the same time, the concentration of accumulated fullerene C_{60} in hepatocytes decreased over time, indicating their ability to be excreted from the rat liver. On the contrary, administration of an aqueous suspension of fullerenes C_{60} at a dose of 2.0 mg/kg body weight to rats for 3, 7, and 14 days before SSl4 poisoning caused a hepatoprotective effect. It is assumed that this effect of fullerenes C_{60} is associated with the prevention of oxidative stress due to their elimination of free radicals and intensification of oxidative modification of proteins against the background of ultra-high values of primary lipoperoxidation products — diene conjugates (DC) and physiological end product — malonic dialdehyde MDA, which is destructive (Jia et al., 2009).

The release of intracellular Ca^{2+}, the activation of Src-kinases, and the phosphorylation of intracellular proteins are thought to be the molecular mechanisms responsible for enhancing blood cell reactivity when influenced by metal nanoparticles. Thus, graphene's high hemocompatibility results from the interplay between its hydrophilic properties and the protection of negative charges provided by hydroxyl and carboxyl groups. This hydrophilic interaction increases under cytotoxic conditions, leading to a concentration of nanoparticles on the cell membrane and subsequent disruption of its integrity.

The LD_{50} of rare earth element (REE) compounds for laboratory animals under single oral administration ranges from 2,000.0 to > 10,000.0 mg/kg body weight, according to literature analysis conducted by Tommasi et al. (2021). These substances can be classified as low-toxic and practically non-toxic substances, falling into Class IV–V in terms of toxicity, and moderately and slightly hazardous substances, falling into Class III–IV in terms of safety, as stated by Klingelhöfer et al. (2020). Therefore, the use of organic nanoforms of REEs is considered an efficient approach due to their low toxicity (Abdelnour et al., 2019) (Cai et al., 2015; Tariq et al., 2020).

Thus, Ou et al. (2000) proposed four possible mechanisms of REE growth-stimulating effects: enhancing enzymatic activity, improving protein metabolism, inhibiting the growth of pathogenic bacteria, and promoting the secretion of digestive fluids into the digestive tract. A little later, the anti-inflammatory and immunostimulatory effects of REE were added to these (Flachowski, 2003), and in 2010, their effect on hormonal activity and increased cell proliferation were identified as possible mechanisms for enhancing the effects of REE (He et al., 2010; Xu et al., 2020). In *in vitro* experiments, $GdVO_4$:Eu^{3+} NPs showed enzyme-like properties: in aqueous solutions, inhibition of superoxide anion formation (similar to the action of superoxide dismutase) and acceleration of hydrogen peroxide decomposition (similar to the action of catalase) were observed (Maksimchuk et al., 2021).

To date, research has found that the colloidal solution of NPs $LaVO_4$: Eu^{3+} displays hydrophobic properties, indicating its aggregation stability in biological fluids, and the potential for it to interact with biomolecules containing a positive charge. Consequently, it can be utilized in both *in vitro* and *in vivo* experiments. *In vivo* experiments showed that $LaVO_4$: Eu^{3+} NPs had no significant impact on erythrocyte hemolysis curves, indicating that they did not affect the adaptation of erythrocytes to osmotic damage, regardless of the medium composition. Furthermore, these nanoparticles had no significant effect on erythrocyte osmotic hemolysis (Pakulova et al., 2017). $LaVO_4$: Eu^{3+} nanoparticles did not show any genotoxicity in the *in vitro* system. The number of micronucleated cells did not differ significantly between native cell cultures (without nanoparticles) and those treated with nanoparticles at concentrations of 30, 65 and 130 μg/cm3. However, the exposure to concentrations of 260.0–520.0 μg/cm^3 led to detachment of cells from the surface, rendering it impossible to count the number of cells with micronuclei (Prokopiuk et al., 2023).

Experiments have shown positive effects of gadolinium orthovanadate nanoparticles in the reproductology (Koshevoy et al., 2021).

To date, there is a scarcity of research on the toxicological and biochemical effects of rare earth metal nanoparticles in *in vivo* experiments. It is suggested by some scientists that the primary characteristic of metals in the nanoscale state is their reduced toxicity in contrast to the traditionally used salts of the corresponding metals. Due to their small size, nanoparticles are capable of directly penetrating and distributing throughout the body through the skin, respiratory and digestive organs, cell membrane openings, or cellular transport mechanisms (Raju et al., 2018; Friedman et al., 2021). Currently, some experimental data has been gathered on the toxicological properties of particular MeNPs through inhalation and oral routes of entry into the macroorganism and, to a lesser extent, through intramuscular, intravenous, and subcutaneous administration.

When studying the adverse effects of inhalation of some nanoparticles into the human body, it was found that inflammatory lung tissue damage is mainly caused by their prooxidant and genotoxic effects (Lam et al., 2004; El-Ansary and Al-Daihan, 2009).

Most of the very small nanoparticles (1 nm), when inhaled, penetrate through the mucosa or paroxysmally through nerve fibers to tissues, are absorbed into the bloodstream, and within 2–4 hours are found in the liver, kidneys, brain, and bone marrow. As a result of transcytosis, nanoparticles enter the blood and lymph through respiratory epithelial cells and sensory nerve endings (Lu et al., 2014; Szewczyk et al., 2022).

It has been shown that polymeric composites of Fluorine nanoparticles used to deliver hormones and bronchodilators circulate in the circulatory system, internal organs and bones, especially in the area of their growth, after 2–6 hours due to the element's tropism to osteoblasts. Additionally, their impact on the hemostatic system, including the development of coagulopathies and thrombosis, has been observed (Nemmar et al., 2002).

The studies described the results of investigating the inhalation toxicity of various nanoparticles including Argentum, Ferrum, Cadmium, Zinc, Titanium, Vanadium, Copper, and Silicon. The studied focused on determining the toxicity of NPAg, with particles ranging from 19.8 to 64.9 nm, in rats exposed to inhalation for a period of 28 days. Three different concentrations were used, with particle/ml concentrations of 1.73×10^4, 1.27×10^5, and 1.32×10^6 (Sahoo et al., 2007). A noteworthy escalation in the activity level of γ-glutamyl transpeptidase (GGT), along with an increase in neutrophils, eosinophils, and hemoglobin levels infemale rats , as well as calcium and total protein levels in the blood serum of male and female rats at a concentration of 1.27×10^5 particles/ml, was identified. There was also an observation of NPAg accumulation in the lungs, liver, and kidneys, and their penetration into the brain via axonal transport. The high stability of NPAg in the environment in terms of retention of toxic properties for several years was proved.

Inhalation of $NPTiO_2$ sized between 80 and 100 nm has been shown to increase the distribution of $NPTiO_2$ in the lungs, elevate the count of neutrophils and phagocytes, and stimulate the formation of proinflammatory cytokines (interleukin 1 (IL-1) and tumor necrosis factor alpha (TNF)) in bronchoalveolar lavage (Ferin and Oberdörster, 1985; Driscoll et al., 1990; Bermudez et al., 2004), which indicates a pronounced prooxidant effect, inflammation and leads to lung fibrosis (Bermudez et al., 2002).

Single oral administration of 25 and 80 nm $NPTiO_2$ to mice at a dose of 5,000 mg/kg body weight resulted in greater toxicity as characterized by intensified penetration into the lungs, liver, spleen, and kidneys, as well as pronounced hepatotoxicity and nephrotoxicity when compared to larger nanoparticles (155 nm) (Warheit et al., 2005; Wang et al., 2007). For instance, the blood serum of animals showed increased activity of lactate dehydrogenase (LDH) and α-hydroxybutyrate dehydrogenase (25 nm), and the animals had liver enlargement and hepatocyte necrosis (80 nm). $NPTiO_2$ has a long half-life (over 500 days) whether inhaled or orally ingested, and is not eliminated through the kidneys.

A comparative study of acute pulmonary toxicity induced by 3 and 20 nm $NPTiO_2$ revealed early biochemical changes in the bronchoalveolar fluid of mice (Bermudez et al., 2002, 2004). After just three days of inhalation, an increase in the content of total protein and alkaline phosphatase (ALP) activity was detected at high concentrations of nanoparticles (40 mg/kg body weight), and at low concentrations (4 mg/kg body weight) — only an increase in ALP, indicating less severe inflammation and no signs of cytolysis and acute pulmonary toxicity.

The impact of subcutaneous administration of 50–60 nm sized NPCu at a 0.05 mg/kg body weight dose on white mice for 3 days has also been described (Strode, 2012). It was found that, in the absence of statistically significant changes in alanine aminotransferase (ALT) activity, there was an elevation in the activity of other enzymes with intracellular localization, including aspartate aminotransferase (AST) and creatine phosphokinase, compared to their control values.

It has been established that the toxicity of MeNPs in the calculation of the maximum biocompatible dose, LD_{50} and LD_{100} depends on their size and concentration, but differs in the mechanism of development from that of metals in macrodispersed form (metal salts).

Water-insoluble nanoparticles with a size less than 20 nm are considered toxic (Hoet et al., 2004; Kagan et al., 2005; El-Ansary and Al-Daihan, 2009), as they can penetrate the body by inhalation, *per os*, through damaged skin, and during injections of drugs with nanocarriers.

Inflammatory reactions, enhanced blood clotting, and the development of coagulopathies all contribute to the detrimental impact of atmospheric nanoparticles smaller than 2.5 nm on the cardiovascular system (Yacobi et al., 2007). Nanoparticles can undergo transcytosis through epithelial and endothelial cells, spread along dendrites and axons of nerve cells, circulate in blood and lymphatic vessels, and exhibit tropism for certain tissues (Moghimi et al., 2001; Müller and Keck, 2004).

In a few studies, it was proved (Abraham and Himmel, 1997) that NPAu with a size of 0.5 to 100 nm are non-toxic, since Aurum is an inert metal in biological systems. In most cases, the formulation of colloidal Aurum is a sol (suspension or dispersion in a liquid phase), and researchers prefer ultracolloidal systems in the form of metal nanoparticles up to 30 nm.

There are data on the size-dependent distribution of NPAu in many organs and tissues of the body, such as the liver, spleen, kidneys, heart, lungs, thymus, genitals, soft tissues, and even the brain and skeleton, when administered intravenously, intratracheally, or orally (Hussain et al., 2001).

The implementation of NPAu research for biomedical purposes faces several challenges despite its prospectivity. The reproducibility of NPAu poses a significant issue (Thaxton et al., 2006), along with the need to comply with GMP requirements for their production (Smith and Korgel, 2008). Additionally, the toxicological aspect of NPAu introduction into clinical practice remains poorly understood. There is a lack of *in vivo* studies, and the mechanism of cell entry and accumulation of MeNPs in the body has not been definitively established (Banerji and Hayes, 2007).

When determining the acute toxicity of AMI-25drug containing NPFe, it was found that the LD_{50} exceeded a dose of 3,000 μmol Fe/kg. Subacute and chronic toxic effects of NPFe were manifested by hemochromatosis if the total amount of metal in the body exceeded 15 g. Since the amount of metal in the dose of the drug is much lower compared to its content in the liver in normal conditions, no significant effect on the total liver Fe concentration was observed (Li et al., 2009).

NPOFe has been found to be a safe treatment option for iron deficiency anemia and as a contrast agent (CA) for magnetic resonance imaging (MRI), with low toxicity to humans except in cases of overdose (Landry et al., 2005; Anzai et al., 2003).

Zhu et al. (2008) investigated the impact of 22 and 280 nm NPOFe on rats, administering inhalation doses of 0.8 and 20.0 mg/kg body weight. The findings revealed the emergence of inflammatory reactions in the lungs, along with ROM induction in cells and disruptions to the blood coagulation system.

In clinical practice, the side effects attributed to NPOFe usage are typically categorized as minor and short-lived. Ferumoxytol and CA AMI-121 administered orally produced hypotension, peripheral edema, and short-term watery diarrhea. Dextran-coated NPOFe preparations were responsible for headache, back pain, vasodilation, and urticaria, which lasted for only one day (Anzai et al., 2003). At the same time, the observed rise in serum iron levels was evidently short-lived, suggesting its absorption.

Inhalation and oral exposure to NPCu in determining acute toxicity revealed a pronounced dependence of the toxic effect on particle size. It was found that low concentrations of NPCu potentiate the toxicity of other elemental substances when exposed to human lung cells (Meng et al., 2007). When ingested, NPCu shifts the acid-base balance of the blood, which leads to the development of metabolic alkalosis (Galla, 2000; Williams, 1998), degenerative changes in the liver, brain tissue, and kidneys (signs of glomerulonephritis).

It is also believed that the water solubility of nanoparticles has a significant impact on cytotoxicity. It has been noted that water-soluble NPCu are toxic (Meng et al., 2007), causing genetic and morphological changes characteristic of Wilson's disease (Tao et al., 2003).

Other authors argue that non-water-soluble nanoparticles (especially those smaller than 25 nm) are more likely to be toxic (Zhu et al., 2007).

However, it is known that the toxicity of NPCu is 2.5–6.0 times lower than that of metal salts. Cu nanoparticles and ionic particles in a suspension of hydroxypolymethyl cellulose are more toxic than microparticles. Toxicity parameters for rats with oral administration of the metal in various forms have been established: LD_{50} for NPCu is 413 mg/kg; for Cu ionic particles — 110 mg/kg; for Cu microparticles — 5,000 mg/kg body weight, respectively. Biochemical changes in animals administered NPCu K4M orally at a dose of 1,080 mg/kg body weight were characterized by an increase in blood levels of urea, creatinine, total bile acids and alkaline phosphatase (ALP) activity, indicating renal and hepatic dysfunction (Chen et al., 2006).

It has been shown that nanoparticles are able to penetrate cells, bypassing any barriers (including blood-brain and placental barriers), and selectively accumulate in different cell types and cellular structures (Chen et al., 2006; Lam et al., 2004; El-Ansary and Al-Daihan, 2009; Colon et al., 2009). There are many experimental studies on the penetration of micro- and nanosized particles into cells. This phenomenon is associated with the cytotoxic effect of nanoparticles on various cell lines and has been shown for endothelial cells, lung epithelium, gastric epithelium, macrophages, nerve cells, and a number of other cells (Hoet et al., 2004).

It has been determined that the cytotoxic effect of NPs is underpinned by oxidative stress and inflammatory reactions (Kipen and Laskin, 2005; Li et al., 2009; Jia et al., 2009), resulting in the development of hepato- and pulmonary toxicity with signs of cytolytic (necrotic)

reactions. Moreover, accumulation of NPs in the liver, lungs, spleen and kidneys is dose-dependent (Lynchet et al., 2007; Bawa, 2008). The absorption of nanoparticles onto the surface of cell membranes, their interference with cell metabolism, and subsequent degradation produce cytotoxic effects. Therefore, studying the biochemical mechanisms of these processes is necessary to assess the potential hazard and biocompatibility of nanoparticles (Weyermann et al., 2005; Jurišić and Bumbaširević, 2008).

To date, our understanding of the toxicodynamics and toxicokinetics of nanoparticles in the body, as well as their impact on the environment, remains limited. Firstly, it is imperative to gather data on the correlation between the toxicity of nanoparticles and their quantity (dose and concentration) and physicochemical characteristics (size, shape, composition, reactivity, etc.) (Donaldson et al., 2004; Santamaria, 2012). Additionally, exploring the molecular mechanisms of their impact on the body, organs, tissues, cells, and identifying the mechanisms that contribute to the development of long-term toxic effects, as well as determining methods to alleviate their adverse effects, are necessary (Zhu et al., 2008; Romanko et al., 2023).

Conclusions. The biosafety of nanomaterials is a complex and multifaceted issue that demands a comprehensive, science-based approach. Modern environmental and economic factors should be considered in this regard. The EU's nanotechnology policy is based on 'an integrated, safe and responsible approach' (CEC, 2008).

Based on the findings of toxicity and antimicrobial activity studies, metal nanoparticles appear to be a favorable choice as antibacterial agents in developing new disinfectants. However, further measures must be taken to ensure the safe and environmentally friendly use of metal nanoparticles (MeNPs). To achieve this, it is crucial to establish toxicity parameters for MeNPs of various compositions, sizes, and concentrations. These parameters must be compared and evaluated alongside the potential effects of MeNPs on laboratory and target animals (*in vivo*), as well as their antibacterial performance against microorganisms of different strains *(in vitro*).

Further investigation on the impact of NPMe, whether in solid or liquid form, on cells of different organizational levels will contribute to a deeper understanding of their biocompatibility or potential toxic effects. Thus, the investigation of possible hazards associated with the use of metal nanoparticles can be effectively achieved by analyzing the fundamental systemic characteristics of biological systems under both in vivo and in vitro conditions, taking into account various aspects such as physiological, biochemical, immunological, genetic and cytological responses that may be affected by toxic effects.

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Part 3. Biosafety

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DETERMINATION OF FUNGICIDAL EFFECT OF DISINFECTANT 'SANDEZVET' ON SANITARY SIGNIFICANT TEST CULTURES OF MOLD MICROMYCETES OF THE GENUS *ASPERGILLUS* MICH.

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Summary. Studying the properties of new disinfectants is a crucial aspect of disinfectology research. During the investigation of the fungicidal properties of the 'SanDezVet' disinfectant, it was discovered that the recommended concentrations of 0.1% and 0.5%, at a temperature of 20.0 ± 0.5 °C and an exposure time of 60, 120, and 180 minutes, do not inhibit the growth of *Aspergillus fumigatus*, *Aspergillus flavus*, and *Aspergillus niger* test cultures. This conclusion was reached as continuous growth of micromycetes was observed in all drug dilutions. The disinfectant 'SanDezVet' at a concentration of 3.0% displayed fungistatic properties by significantly delaying the growth of experimental test cultures. A 5.0% solution of the disinfectant resulted in a complete delay in growth of museum strains of *Aspergillus fumigatus*, *Aspergillus flavus*, and *Aspergillus niger*. This indicates fungicidal properties when compared to the positive control. Based on the obtained results, it was observed that the optimal exposure times for disinfection measures with 'SanDezVet' are 60 and 120 minutes. 'SanDezVet' can be effectively used for fungal infections at different veterinary facilities

Keywords: *Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus niger*

Introduction. Aspergillosis, an acute and chronic mycosis, is an infectious disease affecting birds, animals, and humans. It is characterized by the formation of fibrinous nodular lesions in the respiratory system and serous membranes. This disease is prevalent worldwide and is known to cause significant economic losses to poultry farms due to high poultry mortality rates ranging from 40–90% (Kuznetsov, 2018; Cadena, Thompson and Patterson, 2016). The primary causative agent of aspergillosis in poultry and mammals is the fungus *Aspergillius fumigatus*. In certain instances, *A. flavus, A. niger*, and *A. nidulans* are responsible for significant outbreaks of aspergillosis in chickens and turkeys. *Aspergillus* fungi are prevalent in the environment as saprophytes and become parasitic when ingested under favorable conditions, thereby acquiring pathogenic properties (Chander, 2018; Sharma et al., 2013).

Favorable sanitary and hygienic conditions in poultry housing and feeding are of primary importance in preventing aspergillosis. The use of veterinary and sanitary disinfection measures is essential in increasing productivity and maintaining sanitary quality of products, raw materials, and animal feed (Kuznetsov, 2018; Cadena, Thompson and Patterson, 2016).

Scientific evidence and practical experience suggest that disinfection measures, including disinfection, disinsection, deratization, and sterilization, have proven to be the most cost-effective, affordable, and reliable means of prevention (Vershniak, 2010).

In the context of socio-economic transformations, the role of disinfection in ensuring human safety and health has significantly increased. Disinfection is a vital component in the comprehensive approach to combating contagious diseases. Due to various reasons, disinfection measures are becoming progressively crucial in preventing and eliminating infections. Inadequate funding and the resulting challenges of maintaining sanitary and antiepidemic protocols are the most significant of these circumstances.

It should also be noted that the microbial background has changed as a result of adaptation to the drugs used. Strains of microorganisms that are resistant to traditional disinfectants are increasingly being identified. Environmental safety issues have also been raised recently. The increase in disinfection should not be accompanied by an increase in the release of hazardous chemicals into the environment. For the optimal solution of the above problems related to the need for disinfection in veterinary practice, modern, highly effective disinfectants and antiseptics are needed. Without modern disinfectants, it is impossible to ensure the necessary sanitary and epidemiological regime and reliable protection against infections in farms (Vershniak, 2010; Bordunova et al., 2021).

In view of the above, the study of the properties of new disinfectants is an important area of disinfectology. Therefore, our goal was to determine the fungicidal properties of the new disinfectant 'SanDezVet' on sanitary significant cultures of molds of the genus *Aspergillus* Mich. The study was conducted in the Laboratory of Toxicological Monitoring, Clinical Biochemistry, Safety and Quality of Agricultural Products of the National Scientific Center 'Institute of Experimental and Clinical Veterinary Medicine'.

Materials and methods. The object of the research was the disinfectant 'SefDezInstru', which was first used in veterinary practice in poultry farming under the name 'SanDezVet'.

The research was conducted using commonly accepted procedures for mycological analysis and guidelines for establishing fungicidal properties and optimal disinfectant regimens with *Aspergillus* Mich. test cultures (Seliber, 1962; Yaroshenko et al., 2009; Semenov, 1990). The species affiliation was determined through a comparison of cultural and morphological characteristics, such as the external features of microbial colonies, growth features, color, shape, consistency of colonies, and the presence or absence of sclerotia and pigment. This was done by referencing the descriptions provided in the microbial identifiers and utilizing museum strains of test cultures (Bilay and Koval', 1988; Pidoplichko, 1972).

Mathematical processing of the results was performed using the methods of variation statistics. To calculate the results of the inoculation, all colonies of test cultures of micromycetes that grew in Petri dishes were counted.

The dishes with continuous growth of test culture spores on more than half of the agar surface area, uneven distribution of colonies on the nutrient medium, and those that could not be counted were not taken into account.

The average number of fungal colonies was determined by repeating the experiment three times using the effective parameters of the disinfectant (concentration, temperature, and exposure) and counting the colonies each time. The results obtained were used to calculate the average number of colonies, draw a series of variations, and determine the median. The highest dilution of the drug was effective, as evidenced by at least three experiments that resulted in the death of 95–98% of the spores in the test culture, while the control spores continued to grow. After testing several concentrations, the minimum concentration with the greatest effect on growth retardation of the test culture under optimal temperature and exposure conditions was recommended for use (Semenov, 1990).

Results and discussion. The fungicidal properties of 'SanDezVet' disinfectant were assessed at the recommended concentrations of 0.1, 0.5, 1.0, 3.0, and 5.0% against the most resilient and hygienically significant test cultures of the *Aspergillus* Mich. genus, including *Aspergillus fumigatus*, *Aspergillus flavus*, and *Aspergillus niger,* standardized by spore count. The exposure duration was 60, 120, and 180 min, while the temperature was maintained at 20 ± 0.5 °C.

The fungicidal properties of 'SanDezVet' at various concentrations (0.1, 0.5, 1.0, 3.0, and 5.0%) on *Aspergillus fumigatus* test cultures are shown in Table 1.

Table 1 — Fungicidal activity of 'SanDezVet' against *A. fumigatus* at a temperature of 20 ± 0.5 °C

'SanDezVet' concentrations, %	Terms for calculating the growth of *A. fumigatus* colonies, days														
	3			5			7			10			14		
	Time exposure, min														
	60	120	180	60	120	180	60	120	180	60	120	180	60	120	180
	Number of colonies that grew, pcs.														
0.1	–	–	–	+	+	+	+	+	+	+	+	+	+	+	+
0.5	–	–	–	+	+	+	+	+	+	+	+	+	+	+	+
1.0	–	–	–	+	+	107	+	+	109	+	+	109	+	+	109
3.0	–	–	–	37	17	–	38	18	–	38	19	–	39	19	–
5.0	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Positive control	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Negative control with nystatin	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

Notes: '–' — no growth; '+' — continuous growth.

The results obtained (Table 1) indicate that exposure of the test culture to 0.1, 0.5, and 1.0% solutions at 20 ± 0.5 °C for 60, 120, and 180 min did not affect the decrease in the number of *A. fumigatus* colonies — continuous growth of the micromycete was observed (Fig. 1A). A 1.0% concentration of the drug solution at 180 min exposure showed insignificant fungistatic properties. Whereas 3.0% solution of 'SanDezVet' showed significant fungistatic properties on the test cultures of *A. fumigatus*. The 5.0% solution concentration resulted in a complete growth delay in the test cultures and exhibited fungicidal properties relative to the positive control.

Table 2 shows the results of the evaluation of the fungicidal effects of the 'SanDezVet' disinfectant on *A. flavus* test cultures at concentrations of 0.1, 0.5, 1.0, 3.0, and 5.0%. The data presented in Table 2 demonstrate that exposing the test culture to 0.1, 0.5, and 1.0% solutions for 60, 120, and 180 min at 20 ± 0.5 °C did not have any significant effect on decreasing the number of *A. flavus* colonies; instead, we observed a continuous growth in the micromycete as illustrated in Fig. 1B.

The 1.0% concentration affected the number of colonies that grew after exposure for 180 min, i.e., it showed insignificant fungistatic properties. At 3.0% concentration, 'SanDezVet' solution inhibited the growth of the test culture of *A. flavus*, indicating its fungistatic properties. When using a 5.0% solution, the drug 100% inhibited the growth of the test culture and showed fungicidal properties compared to the positive control.

The results of determining the fungicidal properties of the disinfectant 'SanDezVet' at concentrations of 0.1, 0.5, 1.0, 3.0 and 5.0% on the test culture of *A. niger* are presented in Table 3.

The results obtained indicate that exposure of the test culture to 0.1, 0.5, and 1.0% solutions at (20.0 ± 0.5) °C for 60, 120, and 180 min did not affect the growth retardation of *A. niger* colonies — continuous growth of the test culture was found (Fig. 1C).

The 1.0% concentration at 180 min exposure showed insignificant fungistatic properties — a decrease in the number of colonies was observed.

'SanDezVet' in 3.0% concentration influenced a significant delay in the growth of the test culture of *A. niger* — fungistatic properties were manifested, and 5.0% solution contributed to a complete delay in the growth of the test culture, i.e. it showed fungicidal properties compared to the positive control.

Thus, analyzing the results of Tables 1–3, it should be noted that the recommended concentrations of 0.1–1.0%, temperature parameters 20.0 ± 0.5 °C for exposure time of 60, 120, and 180 min did not affect the growth retardation of test cultures of *A. fumigatus, A. flavus, A. niger*, since in all dilutions of the preparation there was a continuous growth of micromycetes, 'SanDezVet' at 3.0% concentration showed fungistatic properties, and 5.0% — fungicidal properties — complete growth retardation of museum strains of test cultures in comparison with the positive control.

Table 2 — Fungicidal activity of 'SanDezVet' against *A. flavus* at a temperature of 20 ± 0.5 °C

'SanDezVet' concentrations, %	Terms for calculating the growth of *A. flavus* colonies, days														
	3			5			7			10			14		
	Time exposure, min														
	60	120	180	60	120	180	60	120	180	60	120	180	60	120	180
	Number of colonies that grew, pcs.														
0.1	–	–	–	+	+	+	+	+	+	+	+	+	+	+	+
0.5	–	–	–	+	+	+	+	+	+	+	+	+	+	+	+
1.0	–	–	–	+	+	91	+	+	95	+	+	99	+	+	99
3.0	–	–	–	28	10	–	29	11	–	30	13	–	31	15	–
5.0	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Positive control	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Negative control with nystatin	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

Notes: '–' — no growth; '+' — continuous growth.

Table 3 — Fungicidal activity of 'SanDezVet' against *A. niger* at a temperature of 20 ± 0.5 °C

'SanDezVet' concentrations, %	Terms for calculating the growth of *A. niger* colonies, days														
	3			5			7			10			14		
	Time exposure, min														
	60	120	180	60	120	180	60	120	180	60	120	180	60	120	180
	Number of colonies that grew, pcs.														
0.1	–	–	–	+	+	+	+	+	+	+	+	+	+	+	+
0.5	–	–	–	+	+	+	+	+	+	+	+	+	+	+	+
1.0	–	–	–	+	+	88	+	+	89	+	+	89	+	+	89
3.0	–	–	–	25	14	–	29	16	–	29	18	–	29	19	–
5.0	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Positive control	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Negative control with nystatin	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

Notes: '–' — no growth; '+' — continuous growth.

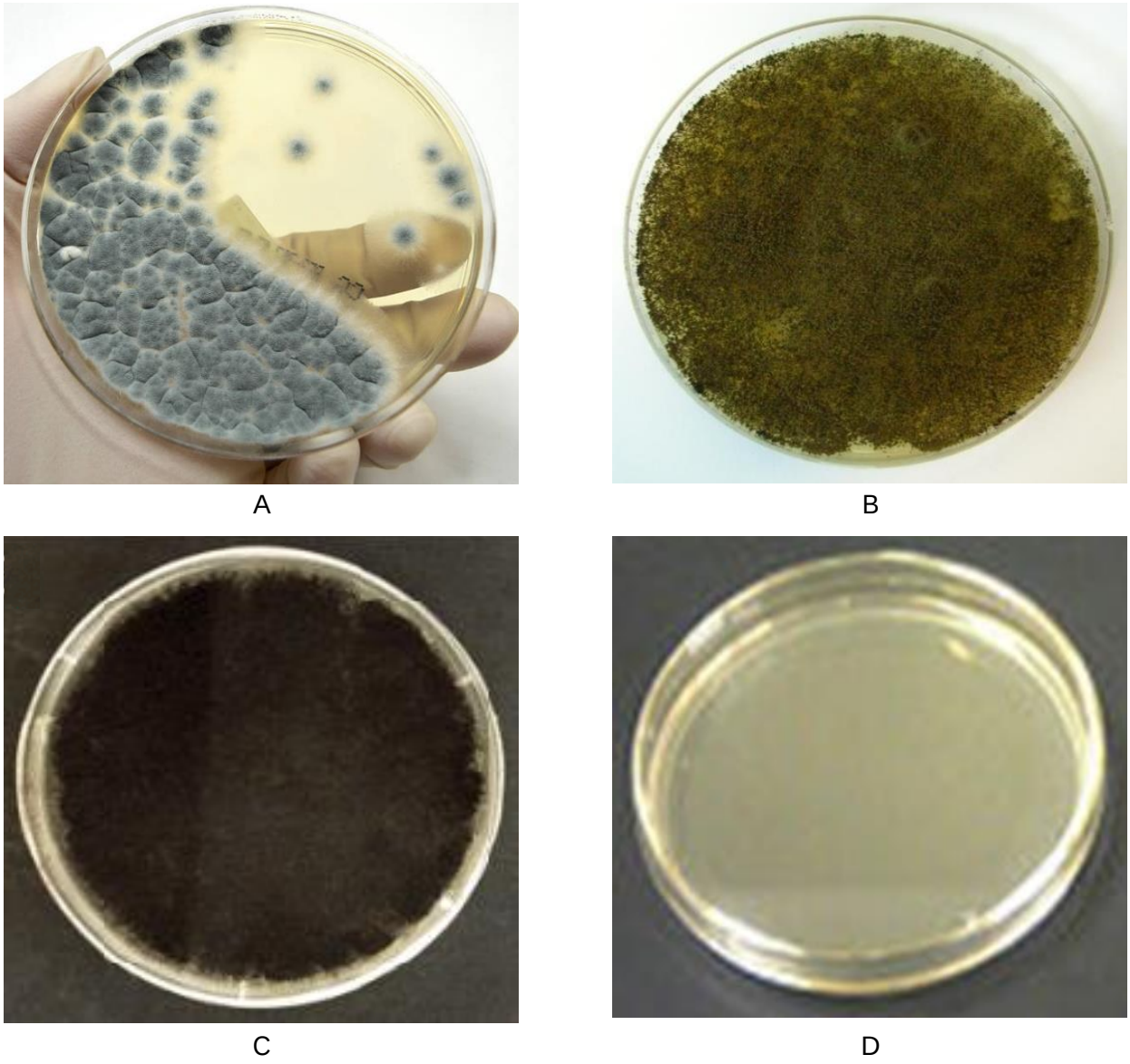

Figure 1. Continuous growth of a 7-day test cultures of *A. fumigatus* (A), *A. flavus* (B), and *A. niger* (C) in 0.5% 'SanDezVet' solution after 180 min exposure compared to the negative control (D).

After analyzing the results, they were statistically processed for the 3.0% solution, the dilution that showed the highest fungistatic properties on all experimental test cultures (Table 4). Comparing the results of the experiments, it should be noted that the most optimal exposure times for 'SanDezVet' disinfection were 60 and 120 min.

Thus, based on the results obtained, 'SanDezVet' can be used for infections of fungal etiology and in different veterinary facilities.

Table 4 — Statistical processing of the results of experiments to determine the fungistatic properties of 3.0% solution 'SanDezVet' against representatives of the genus Aspergillus Mich. at a temperature of 20.0 ± 0.5 °C

Exposure, min	Name of the test culture	Variational range	Average indicator of colonies that have grown	Median
60	*A. fumigatus*	0; 37; 38; 38; 39	30.4	38.0
	A. flavus	0; 28; 29; 30; 31	23.6	29.5
	A. niger	0; 25; 27; 29; 29	22.0	28.0
120	*A. fumigatus*	0; 17; 18: 19; 19	14.6	18.5
	A. flavus	0;10; 11: 13;15	9.8	12.0
	A. niger	0; 14; 16; 18; 19	13.4	17.0

Conclusions. 1. 'SanDezVet' drug in concentrations of 0.1 and 0.5%, at a temperature of 20.0 ± 0.5 °C and exposure for 60, 120, and 180 min had no effect on the test cultures of *Aspergillus fumigatus, Aspergillus flavus, Aspergillus niger* — in all dilutions of the drug, continuous growth of micromycetes was observed.

2. The fungicidal properties of 'SanDezVet' at 3.0% concentration were determined — a significant delay in the growth of experimental test cultures was observed; 5.0% solution of the disinfectant contributed to a complete delay in the growth of museum strains of *Aspergillus fumigatus, Aspergillus flavus, Aspergillus niger* — i.e. it showed 100% fungicidal properties in comparison with the positive control.

Prospects for further research are to study the decontamination concentrations of the disinfectant against museum strains of the test cultures *Penicillium divaricata, Penicillium asymmetrica, Penicillium monoverticillata, Penicillium biverticillata*.

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BACTERIOLOGICAL EXAMINATION OF PET BIRDS' FECES FOR MYCOBACTERIOSIS

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Summary. The article reports findings from a bacteriological study on 232 fecal samples from 29 different companion bird species, searching for mycobacteriosis. The results of the study revealed the detection of atypical mycobacteria in 161 samples, namely *M. scrofulaceum* (n = 3), *M. avium* (n = 4), *M. genavense* (n = 154), which amounted to 1.3, 1.7, and 66.4% of the samples examined, respectively. Co-infections with other pathogens were detected in 62% of the examined fecal samples, independent of a mycobacterial agent's presence. Among these co-infections, *Cryptosporidium* was detected in 34.0% of cases, non-acid-resistant bacteria in 32.6%, and molds and yeast-like fungi in 48.4%

Keywords: *M. avium*, *M. scrofulaceum*, *M. genavense*, *Cryptosporidium*

Introduction. Avian tuberculosis is a very serious disease affecting domestic, exotic and agricultural birds, with pet birds being at increased risk of contracting the disease. There are currently over 130 types of mycobacteria acknowledged, of which ten have been verified as bird pathogens. These mycobacterial species comprise *M. avium* subsp. *avium*, *M. genavense*, *M. tuberculosis*, *M. bovis*, *M. gordonae*, *M. nonchromogenicum*, *M. fortuitum* subsp. *fortuitum*, *M. avium* subsp. *hominissuis*, *M. intracellulare*, *M. scrofulaceum*, and *M. kansasii* (Schrenzel, 2012; Shivaprasad and Palmieri, 2012; Buur and Saggese, 2012).

In a study conducted in Switzerland, *M. genavense* was detected in 71% of cases while *M. avium* complex was only detected in 17%. Other isolates included *M. fortuitum* (4%), *M. tuberculosis* (4%), *M. gordonae* (2%), and *M. nonchromogenicum* (2%) (Hoop, Böttger and Pfyffer, 1996).

Molecular-genetic methods were employed to determine that *M. genavense* was responsible for the majority of mycobacterial infections (up to 80%) in companion birds, particularly within Passeriformes and Psittaciformes species, while MAC was identified in just 5–10% of cases (Tell, Woods and Cromie, 2001; Hoop, 1997; Manarolla et al., 2009).

M. genavense has been found in Coraciiformes, Piciformes, Columbiformes, Ciconiiformes, and Galliformes in previous studies (Tell, Woods and Cromie, 2001; Schmitz et al., 2018a, 2018b), but there have been no reported instances of *M. genavense* detection in large commercial poultry farms.

In birds, *M. genavense* causes a disseminated disease with clinical and histopathological signs that do not differ from the infection caused by *M. avium* (Antinoff et al., 1996; Van Der Heyden, 1997). Nevertheless, a study conducted from 2017 to 2019 observed that mycobacteriosis caused by *M. avium* subsp. *avium* resulted in more common pathological changes, particularly the formation of granulomas. On the other hand, the absence of these signs is more frequently associated with cases caused by *M. genavense* (Schmidt et al., 2022).

In addition to the above two primary avian tuberculosis pathogens, exotic captive birds have been reported with rare infections that result in distinctive pathological organ lesions. These infections are caused by *M. avium* subsp. *hominissuis*, *M. fortuitum*, *M. intracellulare*, *M. scrofulaceum*, *M. gordonae*, *M. celatum*, *M. nonchromogenicum*, and *M. intermedium* (Schmidt et al., 2022; Bertelsen, Grøndahl and Giese, 2006; Kik, Houwers and Dinkla, 2010; Pfeiffer et al., 2017). Furthermore, Psittacine parrots are the sole avian species in which cases of tuberculosis caused by *M. tuberculosis* and *M. bovis* are frequently reported (Schmidt et al., 2008; Washko et al., 1998; Fulton and Sanchez, 2013). Generally, these infections are traced back to human patients with tuberculosis.

The high prevalence of mycobacterial infections among parrots, especially those caused by *M. genavense*, may be due to genetic factors, specific susceptibility of species, as well as exogenous causes (housing conditions, crowding, stress, etc.). In addition, concomitant infections (*Macrorhabdus ornithogaster*, circovirus, polyomavirus, avian bornavirus, adenovirus, *Mycoplasma* species, *Salmonella* species, *Escherichia coli*, *Aspergillus* species and various parasites), by disrupting the immune status, contribute to the activation of *M. genavense* in the host (Manarolla et al., 2009; Schmitz et al., 2018a, 2018b; Manarolla et al., 2007). The danger of mycobacterial infections is that many infected birds often do not show clinical signs of the disease, as mycobacterioses develop slowly and cause a chronic course of the disease, and in most cases in older birds. Due to the high prevalence of mycobacteriosis among domestic parrots and their high susceptibility to mycobacterial infections, purchasing a parrot from an unknown breeder carries a significant risk of infection. The disease is most likely to occur in densely populated areas with poor sanitation and hygiene. Based on the fact that the above mycobacteria are of great

importance in human pathology, domestic parrots can represent a potentially dangerous source of mycobacterial infection for owners, especially for immune compromised individuals, children and the elderly (Realini et al., 1999).

The aim of the study is to determine the species composition of mycobacteria that cause mycobacteriosis in companion birds.

Materials and methods. The research was conducted in 2019–2022. The bacteriological (cultural, bacterioscopic) method was used to analyze 232 fecal samples from companion birds encompassing 29 distinct species of Psittaciformes, Strigiformes, Corvidae, Columbiformes, and Passeriformes. The analyzed samples included 50 from Jaco, 7 — from Cockatoos, 1 — from Suriname parrot, 6 — from Senegalese parrots, 17 — from Amazon (Venezuelan, Cuban, and White-fronted), 3 — from Pyrhurra, 11 — from Ara, 50 — from Corella, and 22 — from Budgerigar. 26 — from Quaker monk, 1 — from Eclectus, 7 — from Alexandrian parrot, 8 — from Cyanoramphus, 4 — from Aratinga, 2 — from rosella, 5 — from lovebird, 10 — from Necklace Parrot, 1 — from Mountain Patagonian, 1 — from Black-capped lory, 1 — from Lord Derby's Parakeet, 1 — from Rüppel's Parrot, 1 — from Amadina, 1 — from Watersluger Canary, 1 — from Eurasian Hobby, 1 — from Eurasian Scops Owl, 1 — from Owl, 1 — from Eagle Owl, 4 — from Crow (gray and black), 1 — from Starling, 4 — from Pigeon. Fecal samples were collected in sterile plastic containers by bird owners. Samples were stored in a refrigerator at the temperature of 4 °C for no more than one day, after that pre-inoculation treatment was performed.

Pre-inoculation treatment of excrement. 1.0 g of excrement was added to a plastic container with 20.0–25.0 cm^3 of sterile distilled water. The mixture was shaken to a homogeneous suspension and settled for 30 min. To a sterile centrifuge tube with 0.08 g of crystalline N-cetylpyridinium chloride, with a sterile pipette 10.0 cm^3 of the supernatant layer of the excrement suspension was added. The tube was hermetically sealed with a rubber stopper, shaken thoroughly until the preparation was dissolved, and left at 18–25 °C for 20 hours. The next day, the tube with the sample was centrifuged for 15 min at 3,000 rpm, the supernatant was removed, and the precipitate was washed once with sterile distilled water by centrifugation (15 min, 3,000 rpm). The supernatant was poured off, and the precipitate was resuspended in 2.0–2.5 cm^3 of 0.85% sodium chloride solution. The suspension was inoculated 0.5 cm^3 into two tubes with nutrient medium for cultivation of mycobacteria and two tubes with nutrient medium with growth factor (mycobactin). The tubes with the cultures were placed in a thermostat at 37–38 °C for 3 days in a horizontal position with not hermetically sealed stoppers, and then paraffinized. Incubation was carried out at a temperature of 37.5–38.5 °C. The presence of colony growth was recorded once a week for 5 months.

The genus identification of *Mycobacterium* and *Cryptosporidium*, tinctorial and morphological characteristics of the detected microorganisms were determined in smears stained by the Ziehl-Nielsen method.

The species affiliation of the isolated mycobacterial culture was determined in biochemical (hydrolysis reaction of Tween-80, amidase and catalase activity, reduction of tellurite) and cultural (growth rate, growth ability at 22 and 45 °C, tolerance to 5% sodium chloride in the medium) tests. To do this, a culture suspension at a concentration of 1.0 mg of bacterial mass in 1.0 cm^3 of 0.85% sodium chloride solution was inoculated onto different media for each test.

Results and discussion. According to the anamnesis, all the birds exhibited diverse health issues including lung and skin lesions, feather loss, joint inflammation, gastrointestinal problems, lethargy, and depression. Furthermore, approximately 50% of the examined birds had positive PCR results for mycobacteria or lung darkening observed on radiographs. Certain birds had bacterial infections as well.

According to study results, 71 (30.6%) fecal samples contained no mycobacteria, while 161 samples contained *Mycobacterium* spp. The number of samples positive for mycobacteria was 69.4%, of which 4.3% (7 cultures) were slow-growing cultures of atypical mycobacteria, namely: 4 non-chromogenic cultures from 2 Jacos, 1 crow, 1 Corella and 3 scotochromogenic cultures from 2 Jacos and 1 Quaker monk. The remaining 95.7% of isolates (n = 154) were identified as *M. genavense*. According to the results of the cultural study, the primary growth of colonies was detected after 25–30 days on both media in the form of round, smooth and shiny white (n = 4) or bright orange (n = 3)colonies. These colonies subsequently merged to form a continuous growth over the entire surface of the medium, as shown in Figs 1, 2.

Microscopy of isolated cultures of microorganisms showed straight or slightly curved, with grains at the poles, acid-resistant rods, which were located individually or in clusters (Figs 3, 4).

According to the Runyon classification, 4 nonchromogenic cultures of atypical mycobacteria were assigned to group III, and 3 scotochromogenic cultures were assigned to group II.

When determining the species affiliation of isolated mycobacterial cultures, it was found that the optimal temperature for colony growth was 37–38 °C, at 22 °C the growth of colonies was slower and less intense, at 45 °C no growth of orange cultures was observed, and nonchromogenic cultures grew, but less intensively.

Figure 1. Growth of nonchromogenic (Corella, Jaco) and scotochromogenic cultures (Jaco 1, Quaker).

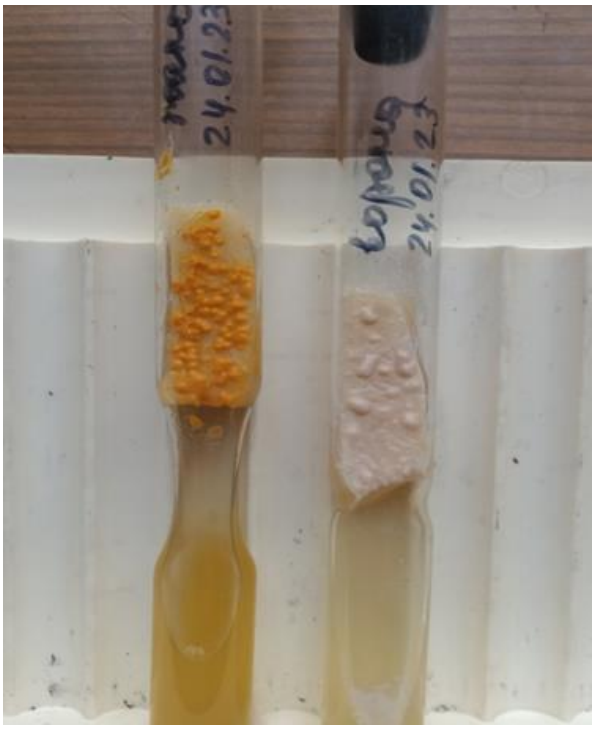

Figure 2. Growth of scotochromogenic culture (Jaco 2) and a nonchromogenic culture (crow) on Pavlovsky's medium.

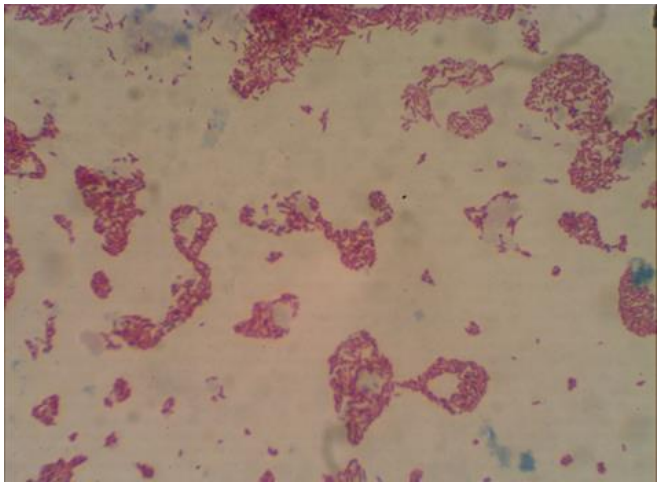

Figure 3. Microscopy of a nonchromogenic mycobacterial culture.

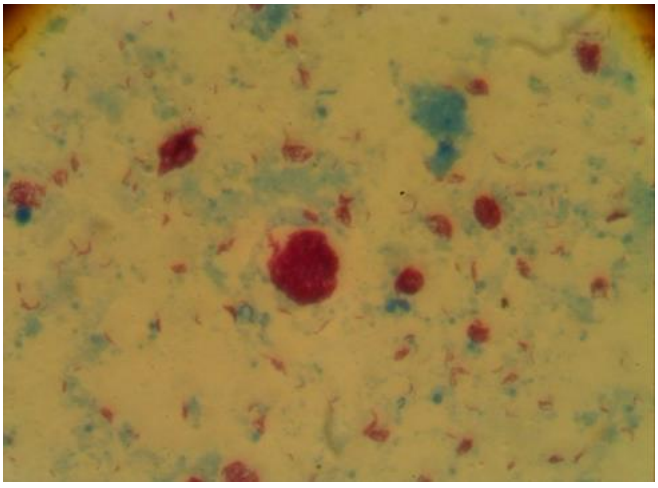

Figure 4. Microscopy of a scotochromogenic culture of mycobacteria.

None of the cultures grew on medium containing 5% NaCl. When determining enzymatic activity in scotochromogenic mycobacterial cultures, positive reactions to nicotinamidase, pyrisinamidase, carbamidase, and catalase were found. The cultures did not reduce tellurite from potassium tellurite and did not hydrolyze Tween-80.

Based on cultural, morphological and biochemical characteristics, 3 scotochromogenic cultures were identified as *M. scrofulaceum*.

It should be noted that mycobacteriosis caused by *M. scrofulaceum* caused death in 2 Jaco parrots. This mycobacterial species is an environmental organism and is frequently present in water and soil, as well as in potable water. The data on the potential of *M. scrofulaceum* to cause infection in birds are debatable. Bertelsen et al. (2006), Hoop et al. (1996), Kik et al. (2010), Pfeiffer et al. (2017) and Abd El-Ghany (2022) have reported that *M. scrofulaceum*, among other non-tuberculous mycobacteria, can cause avian tuberculosis-like pathological lesions in poultry. Marco et al. (2000) found *M. scrofulaceum* in an aviary housing exotic birds. However, their data indicates that *M. scrofulaceum* is not a recognized source of mycobacterial infection (Marco,

Domingo and Lavin, 2000). However, the death of two out of the three birds, namely the Jaco parrots, from which *M. scrofulaceum* was isolated, contradicts this finding. Further data is required to confirm the pathogenicity of this species in parrots.

Regarding nonchromogenic mycobacterial cultures (n = 4), the results of culture, morphological and biochemical tests showed that they had positive reactions to nicotinamidase and pyrisinamidase, restored tellurite on the 9th day, had negative reactions to catalase, carbamidase and hydrolysis of Tween-80. These findings led to the identification of these cultures as *M. avium*. Once diagnosed, the birds were euthanized by their owners. Unfortunately, no necropsies were conducted, resulting in a lack of pathological lesion data.

In the subsequent cultural examination of the remaining samples after 5 months of cultivation in growth factor medium (pH = 6), 154 (66.4%) samples showed very small, transparent, nonphotochromogenic disgonal colonies measuring less than 0.5 mm in size, which did not increase in size regardless of the cultivation period. Microscopy of the scrapings from the surface of this medium or swabs revealed a large number of agglomerates of small short acid-resistant rods (ARR) and cocci (ARC) (Fig. 5), in some cases ARR were found in the cytoplasm of macrophages (Fig. 6).

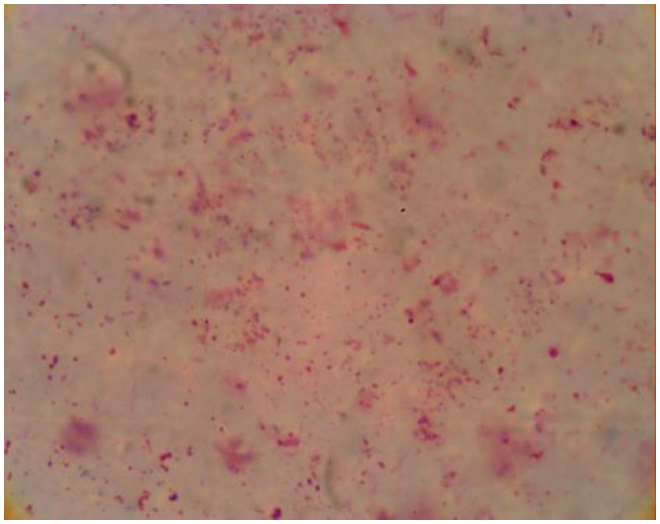

Figure 5. Swabbing off the surface of the medium (Jaco).

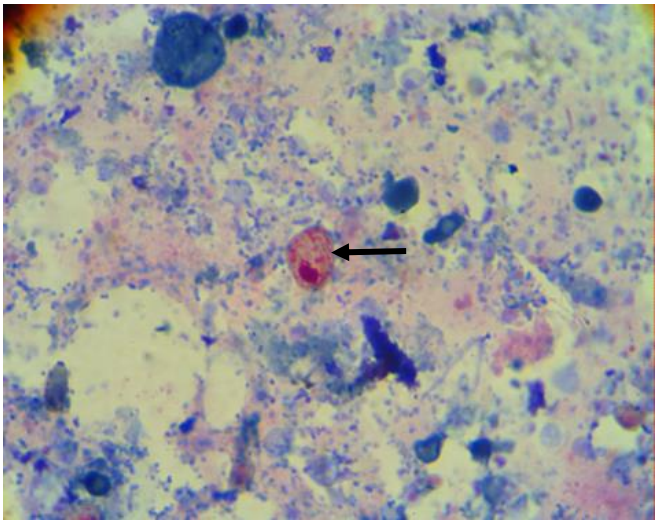

Figure 6. Flushing from the surface of the medium (Cockatoo). Macrophage with ARR and ARC.

The presence of infected macrophages in the feces indicated gastrointestinal inflammation. Under standard cultivation conditions, i.e., on conventional mycobacterial culture medium without growth factor and neutral pH, no colony growth was observed, these microorganisms remained 'unculturable', but microscopy revealed single small clusters of ARR and ARC. In addition, these mycobacteria did not grow on medium with 5% NaCl and at a temperature of 22 °C. Due to 'unculturability' or very long-term growth, it was not possible to obtain a sufficient amount of bacterial mass to study biochemical properties. Based on the results obtained, namely: dependence on growth factor, very slow cell replication and, accordingly, colony growth, cell morphology (small coccoidal forms), tendency to acidic environment (pH = 6.0), and high specific sensitivity of parrots of the genus Psittacinae to this species of mycobacteria, it is reasonable to believe that the detected microorganisms belong to the species *M. genavense.*

It should be noted that a total of 62% of the fecal samples examined revealed concomitant infections caused by other pathogens, regardless of the presence of a mycobacterial agent. Thus, microscopy of mycobacterium-positive smears revealed cryptosporidium co-infection in 15.7% of cases, and cryptosporidium was detected in 18.3% of mycobacterium-negative samples (Fig. 7). Moreover, *Cryptosporidium* was detected at different stages of development in the form of pink spherical oocysts or merozoites. Non-acid-resistant bacteria were detected in 32.6% of the samples, and molds and yeast-like fungi in 48.4% (Figs 7, 8).

Thus, a high prevalence of co-infections indicates a compromised immune status among the studied birds.

During the study, some age dependence was found in the infection of birds with *M. genavense*. The age of the studied birds ranged from 1.3 months to 42 years. But, among the infected birds, the number of birds under the age of 2 years was only 22.1%. The fact that mycobacteriosis in young birds is observed much less frequently was noted by other authors (Tell, Woods and Cromie, 2001; Manarolla et al., 2009; Shivaprasad and Palmieri, 2012).

In addition, among the infected birds, there was a slight correlation between age and the number of bacteria observed during microscopy.

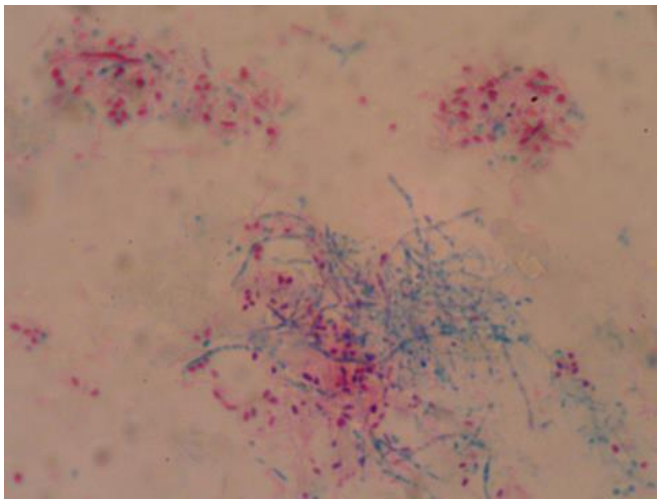

Figure 7. *Cryptosporidium* (pink) and non-acid-resistant bacteria (blue).

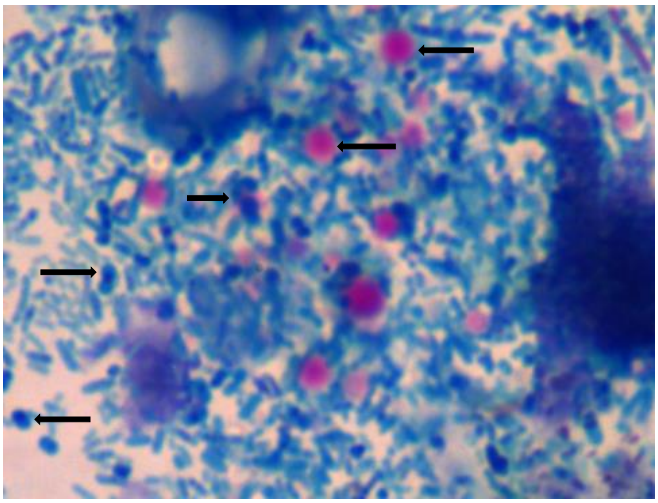

Figure 8. *Cryptosporidium* (oocysts) (pink) and yeast-like fungi (dark blue).

Thus, a higher number of mycobacteria was detected in older birds. It can be assumed that a high coefficient of microscopic detection of mycobacteria indicates the activation of the pathological process. Thus, mycobacteriosis caused by *M. genavense* causes a chronic, latent disease, which is more pronounced in adult birds.

The isolation of *M. avium*, *M. genavense*, and *M. scrofulaceum* suggests that these mycobacterial species circulate and persist among companion birds in Ukraine. Fecal excretion of nontuberculous mycobacteria is a significant mode of transmission for other birds, animals, or humans, directly or indirectly through environmental, feed, or water contamination. Isolated species of mycobacteria, such as *M. scrofulaceum*, are known to cause lymphadenitis, while *M. avium* and *M. genavense* can lead to disseminated infection, particularly in immunocompromised individuals, children, and the elderly.

Close contact with infected birds, whether kept as pets or in zoological gardens, also poses a potential risk of human infection.

Conclusions. The study of feces from companion birds revealed that mycobacteriosis caused by *M. scrofulaceum* was 1.3%, *M. avium* — 1.7%, and *M. genavense* — 66.4%. It is imperative to examine poultry for these mycobacterial species to identify and type the pathogen, considering their potential danger to bird's owners.

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Contents

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