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EFFECT OF ZINC CARBONATE NANOPARTICLES SUBCHRONIC INTAKE ON ANTIOXIDANT STATUS OF MALE RABBITS

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Summary. An urgent scientific task is to develop modern and safe zinc-based nanoparticles that can fulfill rabbits' need for this essential mineral. This research primarily focuses on enhancing the bioavailability of zinc and reducing its toxic properties. To evaluate the antioxidant effect of zinc carbonate nanoparticles, 24 sexually mature male Hiplus rabbits were used, which were randomly divided into two groups of four animals and three replicates each. The rabbits in the experimental group were orally administered zinc carbonate nanoparticles obtained by the co-precipitation method and stabilized with polyvinylpyrrolidone at a dose of 100 mg/kg body weight for 30 days. Animals in the control group received distilled water according to a similar procedure. At the end of the experiment, blood samples were taken from all animals for biochemical studies. The presence of redox activity of these nanoparticles in the organism of male rabbits was established: after administration in the experimental group of animals, a significant decrease in peroxidation markers was noted: the level of thiobarbiturate-active products by 24.5%, diene conjugates by 18.7% and lipid hydroperoxides by 16.2% ($p < 0.05$). A significant increase in the total antioxidant activity (by 40.2%), the content of enzymatic and non-enzymatic components of the antioxidant defense system was confirmed by the expressive antioxidant effect of zinc carbonate nanoparticles: superoxide dismutase activity by 68.9%, catalase by 18.3%, glutathione peroxidase by 27.6%, glutathione reductase by 34.6% and reduced glutathione content by 15.7% ($p < 0.05$). Thus, it can be argued that there is an antioxidant effect of zinc carbonate nanoparticles for male rabbits. The authors of the article consider the study of the effect of these nanoparticles on the state of sexual function in rabbits, as well as pharmacokinetic studies, to be prospects for further research

Keywords: bioavailability, toxicity, blood, biochemical parameters

Introduction. Among the large number of mineral supplements in rabbit diets, zinc (Zn) is one of the most important, especially for males, due to its multifaceted effects, including the ability to improve immune status, digestion, and absorption of nutrients, and the activity of many enzymes (Michalak et al., 2022; Abdel-Wareth et al., 2023b).

The importance of zinc as a cofactor for numerous metalloenzymes plays a leading role in the gastrointestinal tract, metabolism of proteins, fats, and carbohydrates (Malik et al., 2021; Abd El-Hack et al., 2024). The components of the diet used for rabbits (cereals, corn) contain a large amount of phytates, which significantly limit the absorption of zinc and other trace elements (Abdel-Wareth et al., 2020; Abdelnour et al., 2025).

According to Abdel-Wareth et al. (2023a), the average zinc content in rabbit diets is in the range of 30–110 mg/kg without additional addition of this trace element and in the amount of up to 250 mg in diets enriched with 100 mg of zinc oxide in macroform or 60 mg of nanoparticles (NPs).

Recently, there has been a growing interest in using dietary zinc oxide nanoparticles (ZnO-NPs) to enhance performance due to their antioxidant properties, bioavailability, and ability to modulate the rabbit immune system (Abdel-Wareth et al., 2022). ZnO-NPs have both local and systemic antibacterial effects, which have been successfully used in treating wounds in rabbits and other bacterial and surgical diseases in animals and poultry (Awad et al., 2022; Abbas et al., 2023; Obaid et al., 2024). Additionally, an experimental study on rabbits confirmed that ZnO-NPs can accelerate bone growth and mineralization (Zalama et al., 2022; Shokri et al., 2024; Nelogi et al., 2025). Some studies have shown that zinc-based NPs can serve multiple functions — not only as a source of a helpful trace element but also as a therapeutic agent (Hanini et al., 2016). Zinc-based NPs can also be used to develop nanocontainers of theranostic value, as the binding of zinc ions to metallothionein-1X proteins — overexpressed in kidney tumor cells — can increase therapeutic potential, as demonstrated in a rabbit model (Zeng et al., 2025).

However, researchers are mostly interested in the new properties acquired by zinc in the nanoform (Alqahtani, 2025). Thus, on the one hand, there is a decrease in its toxicity compared to inorganic sources, while its bioavailability increases (from 50–60% to 95–99% percent with different routes of administration) (Salimi et al., 2019; Hassan et al., 2024). It is worth noting that nanocrystalline zinc oxide is the most common nanocompound. A large number of studies have focused on ZnO-NPs, including their use as a feed additive for various species of mammals and poultry (Swain et al.,

2016; Fatima et al., 2024). Although the studied dosages of ZnO-NPs *in vivo* models vary significantly (1–1,000 mg/kg b. w.), there are many limitations associated with the introduction of ZnO-NPs into animal husbandry practice (Youn and Choi, 2022; Rahman et al., 2022; Herrera-Rodríguez et al., 2023).

In vivo studies in rats, mice, and rabbits demonstrate the ability of zinc-based NPs to cause toxic effects, such as cyto-, embryo-, nephro-, neuro-, and hepatotoxicity (Bashir et al., 2022; Abouzeinab et al., 2023; Parashar et al., 2024). For example, the LC_{50} of ZnO-NPs is less than 25 μg/ml for rabbit corneal cells in an *in vitro* experiment (Lee and Park, 2019). These toxic effects are mainly caused by high doses (500–1,000 mg/kg b. w.) of ZnO-NPs, with different durations of administration (Fujihara and Nishimoto, 2024; Kahil et al., 2024). Among the main pathogenetic mechanism of their toxicity is oxidative stress, and there are also phenomena of damage to cell membranes, cell mitochondria, DNA, etc. mediated by it (Pei et al., 2023; Serhiienko et al., 2025). Therefore, in recent years, researchers have been developing new methods of synthesis aimed at reducing the toxicity of zinc-based NPs, in particular, using green chemistry methods, organic ligands, etc. (Zhang et al., 2022; Bozer, Dede and Güven, 2024; Elshaer et al., 2025). For example, recent studies of the authors of the article have shown the absence of a general toxic effect of zinc carbonate nanoparticles ($ZnHCO_3$-NPs) synthesised by the co-precipitation method (in doses of 25–200 mg/kg b. w.) on male rats, in particular, hepatotoxic, nephrotoxic, and haematotoxic effects (Naumenko et al., 2023; Koshevoy et al., 2025).

At the same time, there is no data on their redox activity, and therefore it is relevant to study their effect on the antioxidant defense system, which was the **aim** of this study, using model animals — male rabbits, in a subchronic experiment with $ZnHCO_3$-NPs administration of 100 mg/kg b. w. for 30 days.

Materials and methods. The study included 24 adult male rabbits of the Hiplus breed aged 30 weeks and weighing 4.2 ± 0.1 kg. Rabbits for the study were obtained from a private farm located in Kharkiv Region (Ukraine). The animals were kept in the vivarium of the Department of Veterinary Surgery and Reproductology of the State Biotechnological University. Before the experiment, the animals were kept in a preparatory period without the use of pharmacological adaptogens. The concentrated feed and fresh tap water were available *ad libitum* (Tverdokhlib et al., 2024). The rabbits were housed in a well-ventilated room at 25 ± 1°C and with a relative humidity of 55 ± 5% with a regular 12 hours light/12 hours dark cycle (Koshevoy et al., 2022). All animals were randomly divided into two groups: an experimental group and a control group. Each group had 12 animals, with four animals in each group and three replicates. Male rabbits of the experimental group received once a day $ZnHCO_3$-NPs at a dose of 100 mg/kg body weight for 30 days. The control group received the same volume of distilled water.

Synthesis of zinc carbonate nanoparticles and their characterization. Initially, 50 ml of Na_3Cit (Sigma Aldrich, USA) 0.075 M water solution was mixed with 50 ml of $Zn(Ac)_2$ (Sigma Aldrich, USA) 0.1 M water solution. Then, 50 ml of Na_2CO_3 (Sigma Aldrich, USA), 0.15 M water solution was added to the mixture under vigorous stirring. The obtained mixture was heated to 85°C using a water bath for 45 min at constant stirring. The obtained colloidal solution was dialyzed against water for 120 min in a cellulose dialysis sack (pore d = 2.5 nm, MWCO 12,000 kDa). Water was changed every 30 min. The final pH value of the solution was 7.5. Thereafter, 150 ml of 0.6% polyvinylpyrrolidone, used as a stabilizing agent, was added to the solution to obtain 300 ml of zinc carbonate nanoparticles ($ZnHCO_3$-NPs). The final concentration of solid phase ($ZnHCO_3$-NPs) was 2 g/l with 0.3 w% of polyvinylpyrrolidone. Morphology of the as-synthesized $ZnHCO_3$-NPs was analyzed by scanning electron microscopy (SEM, JSM-6390LV, JEOL Company, USA) (Fig. 1a). A TEM image was acquired by a TEM-125K electron microscope (Selmi, Ukraine) using a 100 kV electron beam (Fig. 1b).

Sampling and biochemical methods. Blood samples were taken from the lateral saphenous veins on the 30th day of the study. Pharmacological preparations for sedation and anesthesia of animals were not employed at the time of blood sampling. In the next step, 2 ml of blood samples were taken at the same time throughout the study in tubes with separating gel. The content of OS markers in blood serum was estimated using spectrophotometric methods to determine the concentrations of thiobarbituric acid-reactive compounds (TBA-RC), diene conjugates (DC), and lipid hydroperoxides (LHP). The TBA-RC method is based on the binding of malondialdehyde with thiobarbituric acid to form a stable trimethine complex (wavelength of λ = 532 nm); DC determination is based on the value of the molar extinction coefficient for conjugated dienes of polyunsaturated higher fatty acids (at λ = 233 nm). The concentration of LHP was determined via protein precipitation with trichloroacetic acid and lipid extraction with ethanol. Upon the addition of ammonium thiocyanate to the ethanol lipid extracts, a colorimetric reaction occurred (at λ = 480 nm).

The total antioxidant activity (T-AOC) of plasma was assessed using FRAP analysis (the principle is to determine the antioxidant power of ferrum). A solution of ferric sulfate was used to create a standard curve, and the results were expressed in mmol of Fe^{2+} formed per liter of plasma. Plasma T-AOC was determined spectrophotometrically. Superoxide dismutase activity (SOD) was calculated by the degree of reaction inhibition by the enzyme to reduce nitroblue tetrazolium in the presence of nicotinamide adenine dinucleotide and phenazine methosulfate (at λ = 540 nm). Catalase activity was determined based on the ability of hydrogen peroxide to form a stable complex with ammonium molybdate, color intensity at λ = 410 nm.

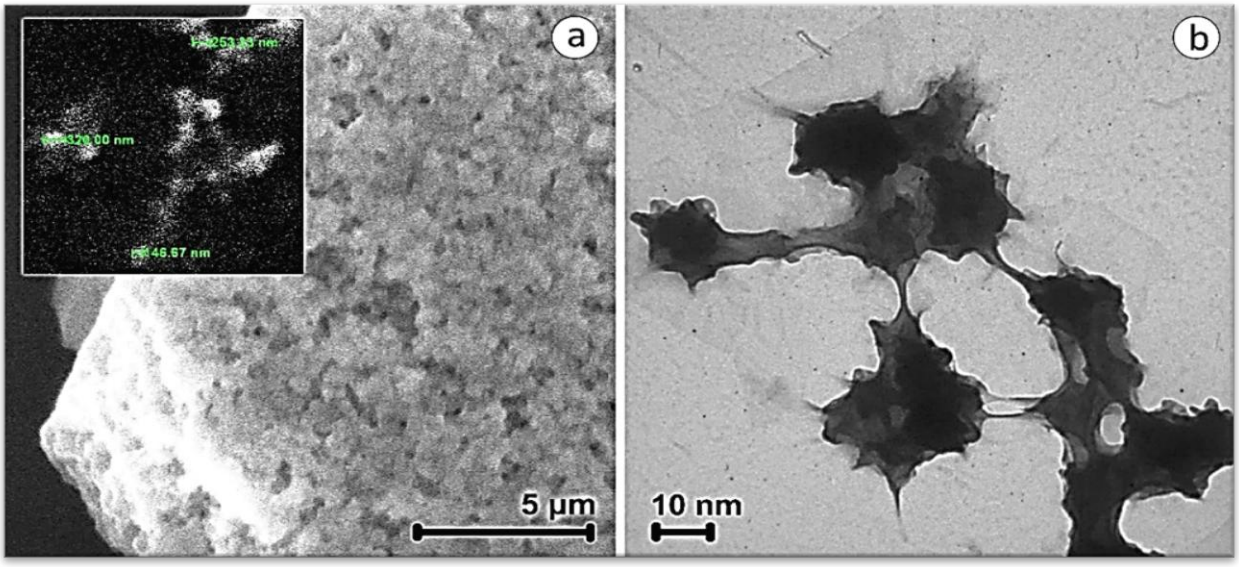

Figure 1. Scanning (a) and transmission (b) electron microscopy images of the synthesized zinc carbonate nanoparticles.

Glutathione peroxidase activity (GSH-Px) was measured based on the oxidation rate of the reduced glutathione in the presence of tBHP in the color reaction with 5,5-dithiobis-2-nitrobenzoic acid (at λ = 412 nm). Glutathione reductase activity (GSH-Rd) was calculated by reducing the content of nicotinamide adenine dinucleotide phosphate at 37°C for 1 min (at λ = 340 nm), and finally, reduced glutathione (GSH) was assessed by the Butler method using Ellman's reagent (at λ = 412 nm). Enzymes and non-enzyme antioxidants were spectrophotometrically determined following Vlizlo (2012).

All manipulations with experimental animals were carried out in accordance with the 'European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes' (CE, 1986) and Council Directive 2010/63/EU (CEC, 2010), and under Art. 26 of the Law of Ukraine No. 3447-IV of 21.02.2006 'About protection of animals from cruel treatment' (VRU, 2006) and basic bioethical principles (Simmonds, 2017). Under the current procedure, the research program was reviewed and approved by the Bioethics Committee of the State Biotechnology University.

Mathematical and statistical analyses were conducted using Statistical Package for the Social Sciences (SPSS), version 22. An ANOVA test was performed to compare data from the control and experimental groups, with normality determined using the Shapiro-Wilk test. Significant differences between groups were confirmed by the Tukey test. Statistical significance was considered to be a P-value less than 0.05.

Results and discussion. The antioxidant effect of subchronic administration of zinc carbonate nanoparticles was assessed by changes in biochemical parameters of male rabbits' blood. First of all, it was important to find out whether the administered dose of $ZnHCO_3$-NPs affected the level of peroxidation products. For this purpose, the content of thiobarbiturate-active products (TBA-RC), diene conjugates (DC), and lipid hydroperoxides (LHP) was evaluated, the results of which are shown in Fig. 2.

In the animals of the control group, the content of TBA-RC was 0.94 ± 0.04 μmol/l, while in the experimental rabbits, it was lower by 24.5% (0.71 ± 0.03 μmol/l, $p < 0.05$). At the same time, there was a decrease in the content of DC by 18.7% (1.09 ± 0.05 μmol/l, $p < 0.05$). Additionally, compared with the control data, the content of LHP decreased to 0.62 ± 0.03 U/l, which was lower by 16.2% ($p < 0.05$). Further, the indicators of the antioxidant defence system were evaluated. First of all, changes in the total antioxidant activity (T-AOC) and the activity of the primary enzymes of the antioxidant defense system — superoxide dismutase and catalase were determined (Fig. 3).

Figure 3 shows that ingesting 100 mg/kg b. w. of $ZnHCO_3$-NPs by male rabbits improved their antioxidant defense. The total antioxidant activity of the blood serum of the experimental rabbits increased by 40.2% compared to the control values (up to 0.861 ± 0.04 mmol×Fe^{2+}, $p < 0.05$). The activity of superoxide dismutase underwent significant changes: if in control rabbits it was at the level of 10.43 ± 0.41 U/mgHb, then after the administration of $ZnHCO_3$-NPs in animals of the experimental group, it increased by 68.9% and amounted to 17.62 ± 0.84 U/mgHb ($p < 0.05$). Less pronounced changes were characterized by catalase activity — in rabbits of the experimental group, it was 18.3% higher than in the control (91.28 ± 2.43 μmol H_2O_2/min×mg protein, $p < 0.05$). We also studied changes in the state of the glutathione link of the antioxidant system under the influence of $ZnHCO_3$-NPs, which are summarized in Fig. 4.

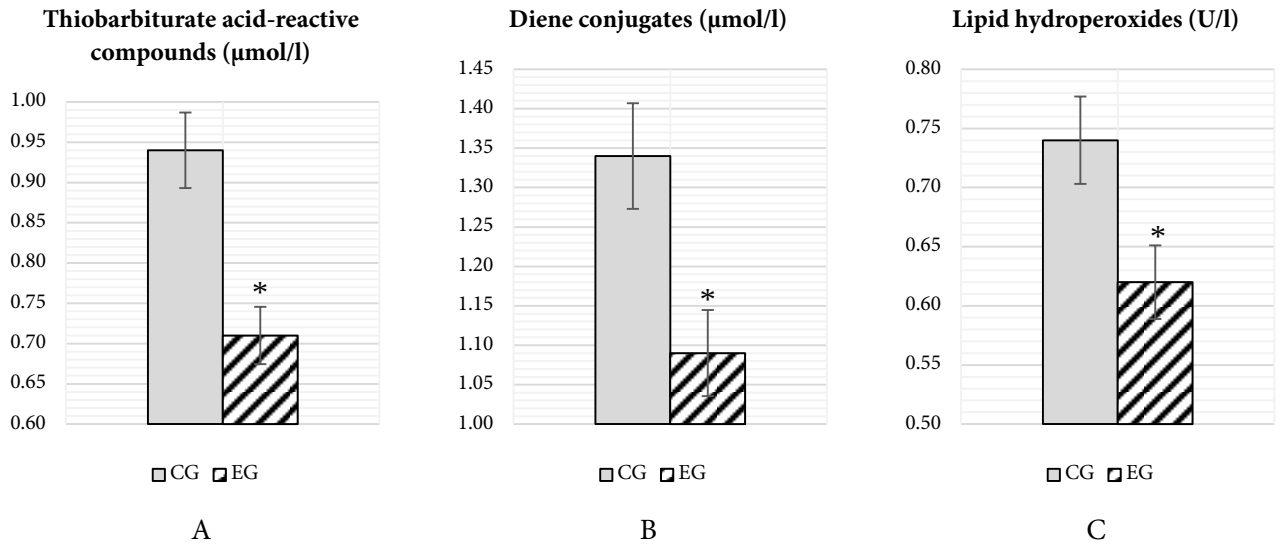

Figure 2. Dynamics of prooxidant system components in male rabbit blood under the action of zinc carbonate nanoparticles: thiobarbiturate acid-reactive compounds (A), diene conjugates (B), and lipid hydroperoxides (C). CG — control group, EG — experimental group. Significant differences ($p < 0.05$) between groups are marked in the figures with *.

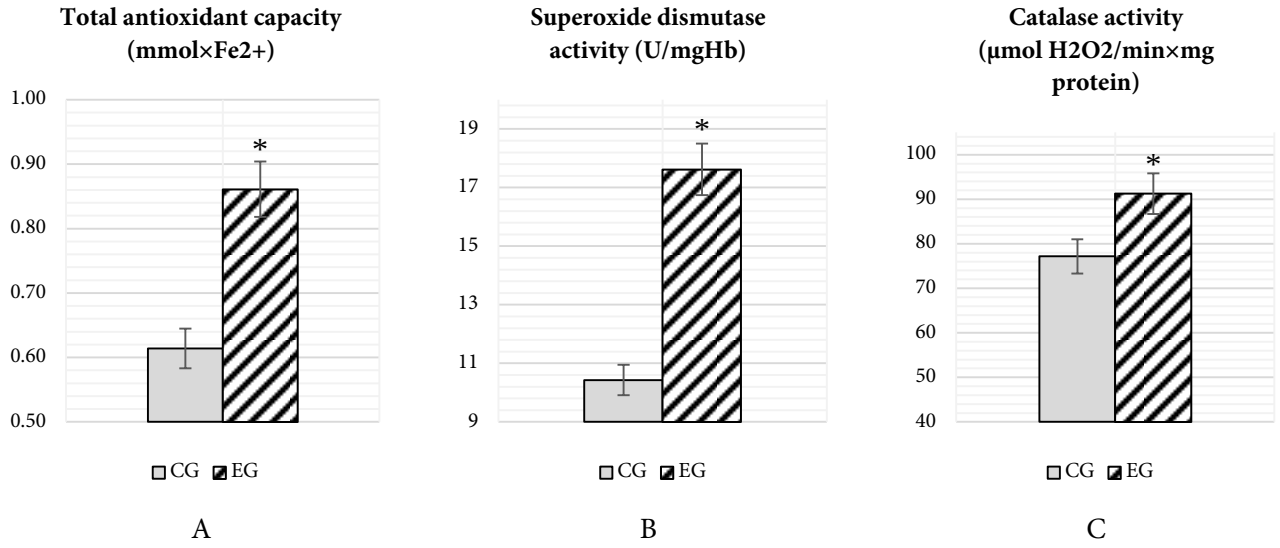

Figure 3. Dynamics of total antioxidant capacity (A), superoxide dismutase activity (B), and catalase activity (C) in male rabbit blood under the action of zinc carbonate nanoparticles. CG — control group, EG — experimental group. Significant differences ($p < 0.05$) between groups are marked in the figures with *.

In general, the obtained changes indicate a partial involvement of the glutathione system. In particular, the content of reduced glutathione increased by 15.7% compared to control rabbits (9.74 ± 0.27 μmol/l, $p < 0.05$). The activity of the enzymes of the thiol-disulfide system underwent more pronounced changes: in the control rabbits, the activity of glutathione peroxidase was at the level of 13.17 ± 0.41 μmol/min×mg protein, and glutathione reductase — 2.14 ± 0.09 μmol/min×mg protein, after the administration of $ZnHCO_3$-NPs, these indicators increased by 27.6% and 34.6%, respectively ($p < 0.05$). Thus, it can be seen that the administration of $ZnHCO_3$-NPs in male rabbits showed a complex antioxidant pharmacological effect — a clear increase in the components of antioxidant defense against a decrease in the level of peroxidation products.

Zinc-based nanoparticles have a multifaceted effect on the rabbit body, which is confirmed by the studies of numerous authors from around the world (Deguchi et al., 2021; Gur et al., 2022; Saha et al., 2024). Such an impact has a diverse manifestation of positive changes — from the health of rabbits to the quality of rabbit products (Halo et al., 2021; Masoud et al., 2025). The study of the effect of zinc oxide-chitosan nanoparticles (ZnO-CNPs) showed the presence of immunomodulatory properties, improved feed intake, total protein, and albumin content in the blood at doses of 50–100 mg/kg of rabbit body weight.

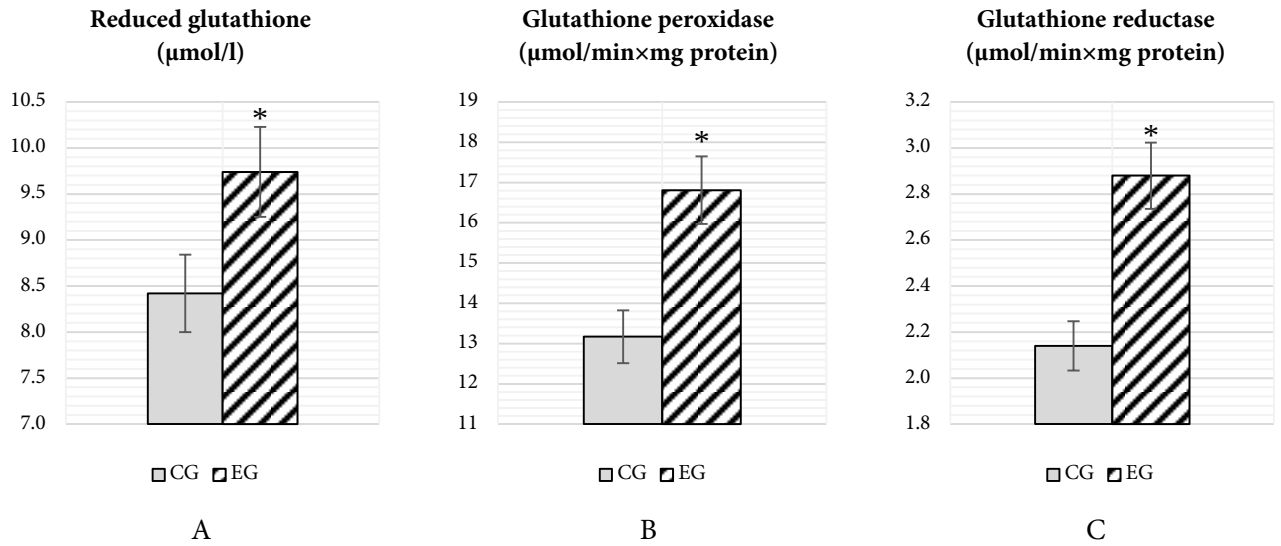

Figure 4. Dynamics of glutathione link of antioxidant system in male rabbit blood under the action of zinc carbonate nanoparticles: reduced glutathione (A), glutathione peroxidase activity (B), and glutathione reductase activity (C). CG — control group, EG — experimental group. Significant differences ($p < 0.05$) between groups are marked in the figures with *.

In addition, a decrease in the content of glucose and total cholesterol in the blood serum of rabbits was observed, indicating the presence of insulin-like effects and antilipidemic properties (Nawaz et al., 2021; Hassan et al., 2023). On the other hand, the use of these NPs is not as widespread as the addition of ZnO-NPs to rabbit diets, which can demonstrate toxic properties at the wrong dosage and depending on the duration of administration to animals (Park et al., 2017; Dahran et al., 2023).

For example, it has been shown that ZnO-NPs can affect liver function in rabbits, contributing to both excessive hepatic enzyme activity and persistent histopathological changes even when administered at low doses of 1–10 mg/kg b. w. (Moasses et al., 2024). The ability of ZnO-NPs at a dose of 60 mg/kg b. w. to reduce the reproductive toxicity of ivermectin in male rabbits, especially to reduce malondialdehyde levels and improve the activity of antioxidant enzymes — superoxide dismutase, catalase, and reduced glutathione levels, was shown (El-Shobokshy et al., 2023). It was also found that the antioxidant system in rabbits treated with 250 µl/kg b. w. improved. ZnO-NPs doped with curcumin in a model of experimental rheumatoid arthritis. Thus, the content of malondialdehyde decreased in rabbits, and indicators of redox processes improved — total antioxidant activity of blood serum, catalase, and superoxide dismutase activity (Azeez et al., 2024).

Different sources of zinc in rabbit diets have different bioavailability and pharmacological activity, so according to Hassan et al. (2021), the use of three different forms of zinc — crystalline zinc oxide as an inorganic source, zinc in combination with methionine as an organic source and zinc oxide nanoparticles — showed the advantage of the organic form and especially nanostructured zinc compared to the usual inorganic macroform. Therefore, a large number of researchers have been studying the synthesis methods, toxicity, and pharmacological properties of zinc-based NPs in recent years (Gomez-Zavaglia et al., 2022; El-Saadony et al., 2024). Taking into account the high toxicity of most zinc-based NPs, the authors of this article have created a new compound — zinc carbonate nanoparticles ($ZnHCO_3$-NPs). Previous studies have shown the low toxicity of these NPs, the absence of death in experimental animals after a single intragastric administration, and confirmed the presence of a positive effect on metabolic parameters (Koshevoy et al., 2023, 2025). At the same time, a large number of pharmacological properties of $ZnHCO_3$-NPs have not yet been investigated.

The results of the study conducted by the authors of this article proved the expressive antioxidant effect of $ZnHCO_3$-NPs on the model of male rabbits. This effect was directed at two main links: the intensity of peroxidation processes and the state of the antioxidant defense system of the rabbit organism. Thus, the data obtained are consistent with the results of El-Shobokshy et al. (2023), in particular, the introduction of $ZnHCO_3$-NPs contributed to a decrease in the content of TBA-RC, DC, and LHP in the body of male rabbits, as well as ZnO-NPs in the above study. In addition, the authors of this article found an increase in the activity of superoxide dismutase and catalase. Similar changes were observed in their studies by Abdel-Wareth et al. (2022) and Azeez et al. (2024) when ZnO-NPs were added to rabbit diets. It is important to note that although the antioxidant effect of $ZnHCO_3$-NPs and ZnO-NPs is similar, their toxicity parameters are different. Concerning $ZnHCO_3$-NPs, their prolonged administration

(30 days) and high dose (100 mg/kg b. w.) did not cause negative changes in the body of rabbits, which was confirmed by a significant increase in the total antioxidant activity. At the same time, we note the presence of a positive effect on the components of the thiol-disulfide link of antioxidant defense — at the end of the experiment, rabbits showed an increase in the activity of glutathione peroxidase and glutathione reductase, as well as the content of reduced glutathione.

According to the authors of the article, it is promising to include $ZnHCO_3$-NPs in the diets of rabbits under heat stress, since there is evidence of improvement of metabolic parameters in such animals under the influence of zinc compounds. For example, the positive effect of ZnO-NPs on the correction of the health status of animals kept under heat stress has been shown, as it is known that its effect leads to oxidative imbalance (Ebeid et al., 2023; Bashar et al., 2024; NasrEldeen et al., 2025). Rabbits treated with ZnO-NPs at a dose of 50 mg/kg b. w. for 60 days had physiological activity of alanine and aspartic aminotransferases, creatinine levels and zinc content in the blood compared to the control group (Abdel-Wareth et al., 2023a; Alrashedi, Almasmoum, and Eldiasty, 2024). The results of the same group of researchers indicate that the addition of ZnO-NPs (20–80 mg/kg) to the diet of rabbits can mitigate the negative effects of heat stress on performance and some biochemical parameters (lipid oxidation, cholesterol, liver enzyme activity) (Abdel-Wareth et al., 2022). It is important to note the positive effect of the introduction of 25–100 mg/kg wt. of ZnO-NPs on the state of androgenesis in male rabbits — it was found that, regardless of the dosage, there was an increase in testosterone levels in animals fed with ZnO-NPs (Abdel-Wareth et al., 2020, 2023b). Given the data obtained by the authors of this article, we consider it promising to further investigate the effect of $ZnHCO_3$-NPs on the state of reproductive capacity of rabbits, the possibility of using them to improve the quality of sperm and its fertilizing ability under conditions of normal temperature or heat stress.

Conclusions. The study of the properties of zinc carbonate nanoparticles under subchronic administration to rabbits by the authors of the article confirmed the hypothesis of their redox activity. Thus, after a 30-day administration of 100 mg/kg b. w. $ZnHCO_3$-NPs in rabbits of the experimental group, a decrease in the intensity of peroxidation processes was observed, which was confirmed by a decrease in the content of thiobarbiturate-active products in the blood serum, the level of diene conjugates, and lipid hydroperoxides. The antioxidant properties of the studied NPs were confirmed by a significant increase in the total antioxidant activity of blood serum, a significant increase in the activity of primary enzymes — superoxide dismutase, and, to a lesser extent, catalase activity. Regarding changes in the state of the thiol-disulfide link of antioxidant defense, the content of reduced glutathione did not increase significantly, and the activity of the enzymes glutathione peroxidase and reductase significantly exceeded the control data.

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EFFECT OF COMBINED THERAPY WITH IMIDOCARB AND PREDNISOLONE ON HEMATOLOGICAL PARAMETERS IN DOGS INFECTED WITH *BABESIA CANIS*

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Summary. *Babesia canis* infection is a significant tick-borne hemoprotozoan disease in dogs, often causing hemolytic anemia and alterations in hematological parameters. This study aimed to evaluate the effect of combined therapy with imidocarb and prednisolone on hematological indices in naturally infected dogs. Thirteen dogs showing initial clinical signs of *B. canis* infection were included in the study. Infection was confirmed via microscopic examination of blood smears. Hematological parameters, including erythrocyte count, hemoglobin concentration, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width (RDW), leukocyte and platelet counts, and total protein, were measured before and after therapy. Treatment consisted of a single simultaneous administration of imidocarb (7 mg/kg) and prednisolone (2.2 mg/kg) within the first 24 hours after diagnosis. Following therapy, significant improvements were observed in red blood cell count, hemoglobin concentration, hematocrit, platelet count, and leukocyte distribution, indicating partial restoration of erythrocyte mass and enhancement of inflammatory and regenerative responses. MCHC, RDW, and total protein showed minimal changes, but overall trends suggest effective alleviation of hemolytic anemia and stabilization of hematological status. The findings support the use of combined imidocarb and prednisolone therapy as an effective early intervention for dogs naturally infected with *B. canis*

Keywords: hemolytic anemia, tick-borne infections, blood

Introduction. Babesiosis is one of the most common transmissible protozoan diseases of dogs, caused by members of the genus *Babesia*, of which *Babesia canis* is the most clinically significant (Birkenheuer et al., 2020; Bajer et al., 2022). The disease is characterized by damage to erythrocytes, leading to the development of hemolytic anemia, thrombocytopenia, leukocyte changes, and a systemic inflammatory response (Boozer and MacIntire, 2003; Eichenberger et al., 2016). Clinical manifestations vary from mild apathy and fever to severe complications such as shock, multiple organ failure, and death (Solano-Gallego et al., 2016; Strobl et al., 2020).

B. canis is transmitted by the bite of infected ticks, mainly *Dermacentor reticulatus*, whose activity is regulated by seasonal and climatic factors (Kohn et al., 2019; Rubel et al., 2020). Given the expansion of the vector range and changing climatic conditions, the spread of babesiosis among dogs in Europe and other regions shows a tendency to increase, making this disease a pressing problem in veterinary medicine (Bajer et al., 2022; Weingart et al., 2023). This expansion is evidenced by reports of canine babesiosis in previously non-endemic areas, underscoring its emerging status (Pawelczyk et al., 2022). Regional studies in Eastern Europe, such as in Kharkiv Region of Ukraine, have demonstrated high levels of *Ixodes ricinus* tick infestation with *Babesia* spp., with infection rates reaching up to 35.8% in adult ticks and a resulting 36.9% infection rate among dogs following tick bites, highlighting the significant local risk (Sumakova et al., 2025).

Modern treatment of babesiosis is based on the use of antiprotozoal drugs, in particular imidocarb dipropionate, which has demonstrated high efficacy in eradicating the parasite (Penzhorn et al., 1995; Baneth, 2018). While other drugs like doxycycline have been explored for prophylactic purposes, their efficacy in treatment is limited, highlighting the need for effective therapeutic protocols (Vercammen, De Deken and Maes, 1996). However, imidocarb does not always prevent severe hematological disorders and can cause side effects, including hepatotoxicity and nephrotoxicity (Kock and Kelly, 1991; Máthé, Dobos-Kovács and Vörös, 2007). Furthermore, the emergence of drug resistance is a growing concern in the control of protozoan parasites, which could potentially compromise the efficacy of existing treatments (De Koning, 2017). In this regard, researchers suggest a combination therapy that includes glucocorticoids, in particular prednisolone, to reduce the inflammatory response and immunopathological damage to the blood (Schoeman and Herrtage, 2008; Sikorski et al., 2010). Previous experimental and clinical studies have shown that the combination of antiprotozoal treatment and corticosteroids can accelerate the recovery of hematological parameters, such as red blood cell, hemoglobin, and platelet levels, and reduce the risk of complications (Reyers et al., 1998; Brandão, Hagiwara and Myiashiro, 2003; Máthé et al., 2006). The immune response to *B. canis* is complex, and understanding the duration of protective immunity following infection or treatment remains a critical area of investigation (Vercammen, De Deken and Maes, 1997). However, most of the existing data are limited to individual clinical cases or experimental models, and there are not enough systematic studies on the effect of combination therapy on the hematopoietic system of dogs with natural infection with *B. canis*.

Therefore, evaluating the effect of combination therapy with imidocarb and prednisolone on

hematological parameters in dogs infected with *B. canis* is of utmost importance to optimize the treatment strategy, reduce the risk of complications, and increase the survival of the animals.

B. canis infection is a significant tick-borne hemoprotozoan disease in dogs, frequently causing hemolytic anemia and alterations in hematological parameters. Previous investigations have documented pronounced reductions in red blood cell count, hemoglobin concentration, and hematocrit in infected canines, reflecting severe anemia due to parasite-driven hemolysis (Boozer and MacIntire, 2003; Bajer et al., 2022). The prevalence and clinical significance of this disease are recognized not only in Europe but also in other parts of the world, as highlighted in regional reviews (Panti-May and Rodríguez-Vivas, 2020). Studies from endemic regions, such as Ukraine, report a high incidence of the disease following tick exposure, with over 46% of dogs developing babesiosis after being bitten by an infected tick, underscoring the substantial burden of the disease in these areas (Sumakova et al., 2025). Studies evaluating therapeutic approaches, particularly the administration of imidocarb, have reported partial restoration of erythrocyte mass and initiation of erythropoietic regeneration (Máthé et al., 2006; Máthé, Dobos-Kovács and Vörös, 2007; Baneth, 2018).

Mild declines in total protein levels have been associated with subtle disturbances in protein metabolism, possibly linked to inflammatory processes and tissue healing (Birkenheuer et al., 2020). Hematological indices such as mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), and red cell distribution width (RDW) in previous studies have demonstrated a microcytic pattern with minimal changes in hemoglobin per erythrocyte, suggesting early regenerative activity and limited variability in red cell size (Onishi et al., 1993; Brandão, Hagiwara and Myiashiro, 2003).

Red blood cell alterations reported in the literature indicate that *Babesia* infection triggers both direct hemolytic effects and compensatory erythropoietic responses. Pre-treatment microcytosis and slightly reduced MCHC may reflect iron redistribution and hindered hemoglobin production due to infection-associated inflammation (Máthé et al., 2006; Birkenheuer, 2021). Gradual restoration of MCV and MCHC following effective therapy has been documented, indicating recovery of erythropoiesis after parasite clearance.

Thrombocytopenia is consistently observed in *Babesia*-infected dogs, reflecting platelet depletion through immune-mediated destruction, splenic sequestration, and direct parasitic effects on megakaryocytes (Scheepers et al., 2011; Strobl et al., 2020). Studies have shown partial restoration of platelet counts following antiparasitic therapy, highlighting the benefit of early intervention.

Leukocyte dynamics in canine babesiosis have also been described, with initial leukopenia and post-treatment neutrophilia accompanied by persistent lymphopenia, reflecting ongoing inflammation and modulation of the immune response (Wykes et al., 2014; Eichenberger et al., 2016; Solano-Gallego et al., 2016). Serial monitoring of these parameters has been emphasized as valuable for prognostic evaluation and guiding supportive care (Solano-Gallego et al., 2016; Weingart et al., 2023).

Finally, regional differences in *Babesia* species and vector ecology have been reported to influence the severity and pattern of hematological alterations, underlining the importance of local epidemiological knowledge in clinical management (Rubel et al., 2020; Bajer et al., 2022). Collectively, these studies underscore the complexity of hematological responses in canine babesiosis and the critical role of prompt and targeted therapeutic interventions (Penzhorn et al., 1995; Baneth, 2018; Weingart et al., 2023).

This study **aimed** to evaluate the effect of combined therapy with imidocarb and prednisolone on hematological indices in naturally infected dogs. To achieve this aim, 13 dogs naturally infected with *B. canis* were included in the study. The following tasks were set: to confirm infection by microscopic examination of blood smears; to study the dynamics of hematological parameters, including erythrocyte count, hemoglobin concentration, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width (RDW), leukocyte and platelet counts, and total protein; to evaluate the effect of combined therapy with imidocarb (7 mg/kg) and prednisolone (2.2 mg/kg) on these hematological parameters.

Materials and methods. A clinical study on the effect of combined therapy with imidocarb and prednisolone on hematological parameters in dogs infected with *B. canis* was conducted. The study included 13 dogs that exhibited the first signs of *B. canis* infection at the time of the initial veterinary consultation, which was confirmed by the results of blood smear analysis. This diagnostic method, involving the microscopic identification of intraerythrocytic parasites on stained blood smears, is a well-established and reliable technique for confirming acute *Babesia* infection, as utilized in other regional studies (Sumakova et al., 2025). The study was carried out from February to April 2024 at the Veterinary Complex 'Peredovyi' in Dnipro (Amur-Nizhnyodniprovsky District). The animals represented various age groups, breeds, and sexes. Dogs with diagnosed concomitant diseases or with incomplete or unreliable laboratory data were excluded from the study. Detection of *B. canis* parasites in erythrocytes was performed using thin blood smears stained with fast-acting LEUCODIF 200 dye (Erba Lachema, Czech Republic), followed by examination under 100× magnification using an optical microscope Leica DM4 (Germany) (Fig. 1). Intraerythrocytic forms of the parasite were identified and documented.

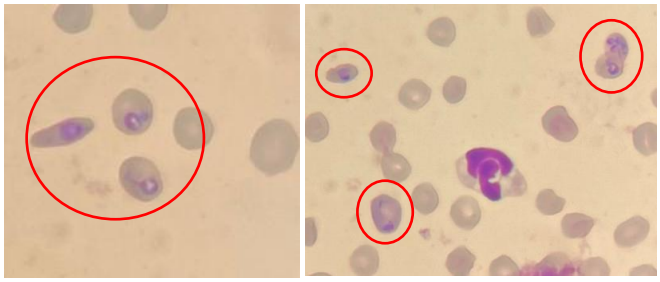

Figure 1. Blood smear from dogs infected with *B. canis* (Leucodiff stain, ×100 oil immersion, NA 1.25). Intraerythrocytic forms of the parasite are clearly visible and indicated with a red circle.

For hematological analysis, blood samples were collected from the cephalic or saphenous vein into EDTA-containing tubes. The following parameters were measured: erythrocyte count, hemoglobin concentration, leukocyte count, platelet count, mean corpuscular hemoglobin concentration (MCHC), erythrocyte distribution width (RDW), total protein, and hematocrit. These parameters were analyzed using an automatic hematological analyzer MicroCC-20 Plus (HTI, USA). Quantitative assessment of segmented neutrophils and lymphocytes was performed by microscopic counting on stained blood smears.

The main period of treatment was the first 24 hours, during which dogs received combined therapy with imidopyran (Arterium, Ukraine, 7 mg/kg) and prednisolone (Darnitsa, Ukraine, 2.2 mg/kg) administered simultaneously. Clinical signs and hematological parameters were monitored at baseline, during treatment, and after completion of therapy.

Animal handling and all procedures were performed following the recommendations of the 'European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes' (CE, 1986) and Council Directive 2010/63/EU (CEC, 2010), and under Art. 26 of the Law of Ukraine No. 3447-IV of 21.02.2006 'About protection of animals from cruel treatment' (VRU, 2006) and basic bioethical principles (Simmonds, 2017). Under the current procedure, the research program was reviewed and approved by the Bioethics Committee of the Veterinary Complex 'Peredovyi' (Dnipro, Ukraine).

A range of statistical methods was applied to evaluate hematological changes associated with the combined therapy. Descriptive statistics were calculated to determine mean, median, mode, standard deviation, and variance for both the control group and the treated group. Differences in mean values were analyzed using the Student's *t*-test, and analysis of variance (ANOVA) was applied to compare mean values across different time points and groups (Van Emden, 2019).

Results and discussion. Key hematological parameters were analyzed in dogs at three stages: clinically healthy (control group), before treatment, and after treatment of babesiosis.

In infected dogs, the RBC (Fig. 2a) count was significantly reduced before treatment to $3.59 \pm 0.37 \times 10^6$/μL ($P < 0.0001$; $F = 0.0107$), indicating pronounced anemia due to parasite-induced hemolysis. Following therapy, the RBC partially recovered to $4.20 \pm 0.27 \times 10^6$/μL ($P = 0.0001$ compared to pre-treatment; $F = 0.2763$), reflecting improvement in hematological status.

Hemoglobin levels (Fig. 2b) also decreased markedly before treatment to 83.42 ± 2.96 g/L ($P < 0.0001$; $F = 0.0250$) and rose to 90.77 ± 3.38 g/L after therapy ($P < 0.0001$; $F = 0.6554$), indicating partial restoration of oxygen-carrying capacity.

Hematocrit (Fig. 2c) followed a similar trend, dropping to 22.54 ± 1.45% before treatment ($P < 0.0001$; $F = 0.0955$) and increasing to 26.92 ± 1.42% after therapy ($P < 0.0001$; $F = 0.9457$), demonstrating improvement in erythrocyte volume fraction.

In infected dogs, the mean total protein level (Fig. 3) was slightly reduced before treatment at 65.59 ± 2.26 g/L ($P = 0.1300$; $F = 0.1845$) and decreased further to 63.06 ± 1.55 g/L after therapy ($P = 0.0038$; $F = 0.2081$), suggesting mild alterations in protein metabolism during infection.

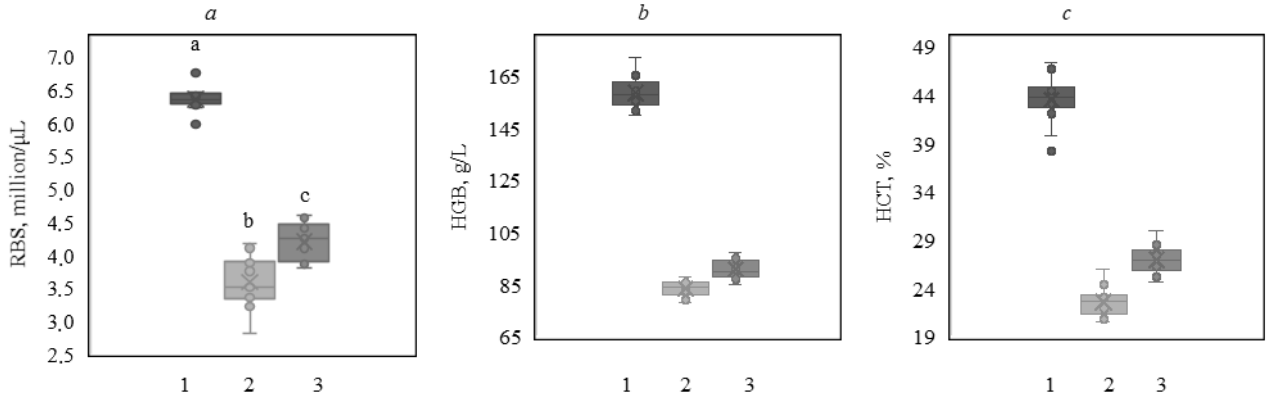

Figure 2. Comparative analysis of erythrocyte indices in dogs: (*a*) red blood cell count, (*b*) hemoglobin concentration, and (*c*) hematocrit values (x ± SD, n = 13): 1 — clinically healthy control dogs, 2 — infected dogs before therapy, 3 — infected dogs after therapy; ^{a,b,c} — mean values with unlike letters were significantly different between the groups ($P < 0.05$).

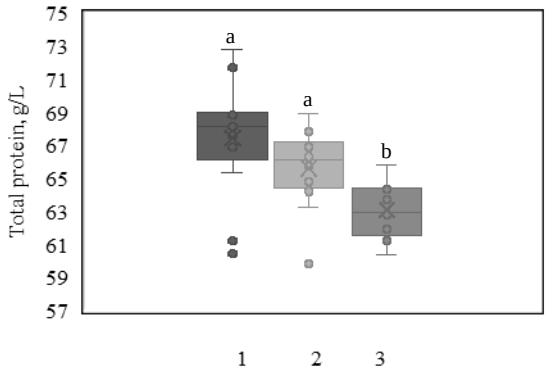

Figure 3. Serum total protein concentrations in control dogs and in animals with babesiosis prior to and following treatment (x ± SD, n = 13): 1 — clinically healthy control dogs, 2 — infected dogs before therapy, 3 — infected dogs after therapy; [a,b,c] — mean values with unlike letters were significantly different between the groups (P < 0.05).

In infected dogs, MCV (Fig. 4a) decreased to 63.45 ± 2.49 fL before treatment (P = 0.0178; F = 0.4335) and slightly increased to 64.69 ± 2.59 fL after therapy (P = 0.2423; F = 0.8904), indicating microcytic erythrocytes and an early regenerative response.

MCHC (Fig. 4b) remained relatively stable, measuring 36.21 ± 5.58 g/dL before treatment and 35.72 ± 6.81 g/dL after therapy (P > 0.05), suggesting limited changes in hemoglobin concentration per erythrocyte. RDW (Fig. 4c) showed minimal variation, with values of 15.99 ± 1.04% before treatment and 15.67 ± 1.05% after therapy (P > 0.05), indicating little change in erythrocyte size distribution.

In infected dogs, platelet count (Fig. 5) dropped sharply to 38.23 ± 6.20×10³/μL before treatment (P < 0.0001; F = 0.0000) and partially recovered to 65.15 ± 4.80×10³/μL after therapy (P < 0.0001; F = 0.3370), highlighting severe thrombocytopenia during acute infection and improvement following treatment.

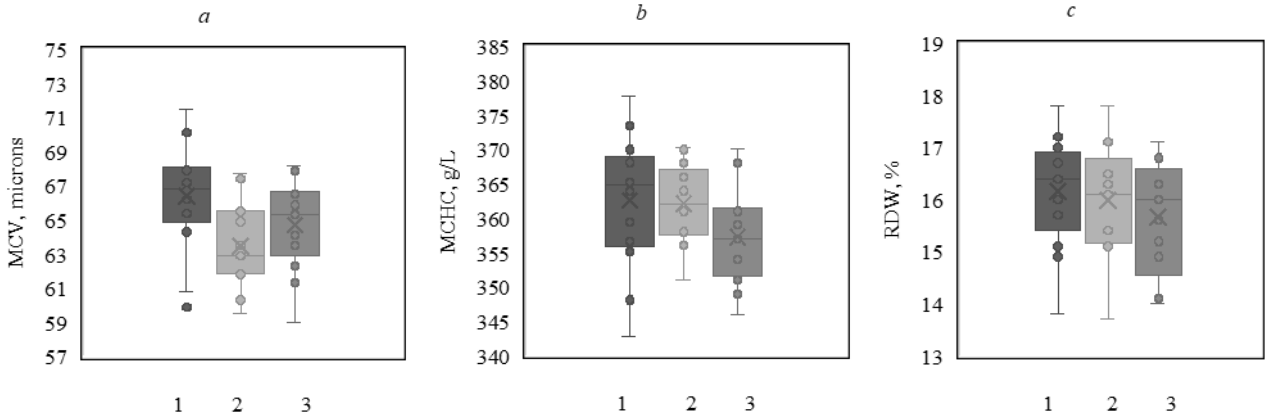

Figure 4. Erythrocyte indices in dogs naturally infected with *B. canis*: (*a*) mean corpuscular volume (MCV), (*b*) mean corpuscular hemoglobin concentration (MCHC), and (*c*) red cell distribution width (RDW) (x ± SD, n = 13): 1 — clinically healthy control dogs, 2 — infected dogs before therapy, 3 — infected dogs after therapy; [a,b,c] — mean values with unlike letters were significantly different between the groups (P < 0.05).

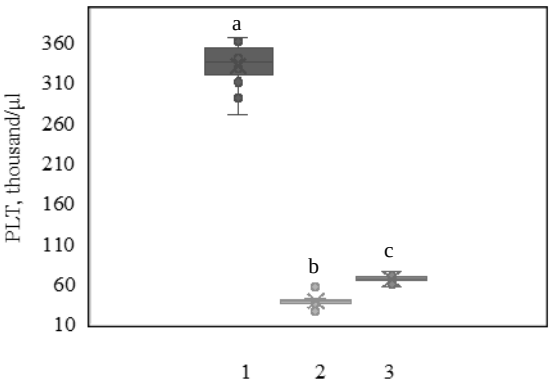

Figure 5. Platelet counts (PLT) in control dogs and in dogs infected with *B. canis* before and after treatment (x ± SD, n = 13): 1 — clinically healthy control dogs, 2 — infected dogs before therapy, 3 — infected dogs after therapy; [a,b,c] — mean values with unlike letters were significantly different between the groups (P < 0.05).

In infected dogs, WBC count (Fig. 6a) decreased to 7.08 ± 0.60×10³/μL before treatment (P < 0.0001; F = 0.0676) and increased to 11.68 ± 0.79×10³/μL after therapy (P < 0.0001; F = 0.6319).

Segmented neutrophils (Fig. 6b) rose from 60.66 ± 1.88% before treatment to 73.66 ± 1.63% after therapy (P < 0.0001; F = 0.0688), indicating an ongoing inflammatory response.

Lymphocytes (Fig. 6c) decreased from 28.45 ± 1.27% before treatment to 25.68 ± 1.27% after therapy (P < 0.0001; F = 0.4798), reflecting shifts in leukocyte populations during infection and recovery.

These findings are largely consistent with previously published studies on canine babesiosis. Similar improvements in red blood cell count, hemoglobin concentration, hematocrit, and platelet levels following combined therapy with antiprotozoal drugs and corticosteroids have been reported by Máthé et al. (2006), Brandão, Hagiwara and Myiashiro (2003), and

Reyers et al. (1998), indicating that early intervention with imidocarb and prednisolone effectively supports hematological recovery. The observed stability in MCHC and RDW aligns with the findings of Onishi et al. (1993) and Birkenheuer (2021), suggesting that regenerative erythropoiesis restores red cell mass without marked alterations in cell size or hemoglobin concentration per erythrocyte. Additionally, the partial restoration of leukocyte counts and neutrophil predominance after treatment corresponds with the immune modulation patterns described by Solano-Gallego et al. (2016) and Eichenberger et al. (2016). Overall, our results reinforce the evidence that combination therapy not only alleviates hemolytic anemia but also helps normalize inflammatory and immune responses in dogs naturally infected with *B. canis*.

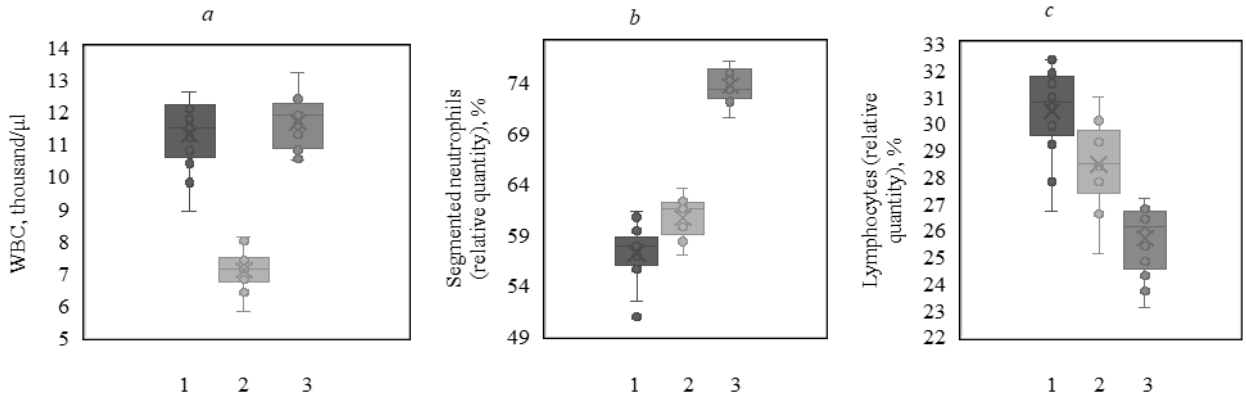

Figure 6. (*a*) Total white blood cell counts (WBC), (*b*) Relative segmented neutrophils, and (*c*) Lymphocyte proportions in control dogs and dogs infected with *B. canis* before and after treatment (x ± SD, n = 13): 1 — clinically healthy control dogs, 2 — infected dogs before therapy, 3 — infected dogs after therapy; ^{a,b,c} — mean values with unlike letters were significantly different between the groups ($P < 0.05$).

Conclusions. Within the first 24 hours of combined therapy with imidopyran (7 mg/kg) and prednisolone (2.2 mg/kg), infected dogs showed early signs of hematological and biochemical recovery. Compared to pre-treatment values, there was a notable increase in platelet and white blood cell counts, as well as the beginning of regenerative changes in erythrocytes. Despite these improvements, some parameters, including lymphocyte percentages and total protein levels, remained below those of clinically healthy controls, indicating ongoing systemic effects of infection. These findings demonstrate that prompt administration of imidopyran and prednisolone can rapidly mitigate acute manifestations of babesiosis, although continued monitoring is essential to ensure complete recovery.

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COMPARATIVE ANTIPARASITIC EFFECTIVENESS OF THE COMBINED DRUG AND NIFULIN IN THE TREATMENT OF BLASTOCYSTOSIS AND HISTOMONIASIS IN TURKEYS

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Summary. For decades, effective drugs have been used to prevent and treat protozoosis in Europe, the United States, and other countries. However, these drugs were subsequently withdrawn from use for consumer safety reasons. Since then, research has focused on finding alternative approaches, ranging from new chemotherapeutic agents to plant-based compounds. Using phytogenic substances in turkey feed is considered promising because they are effective and act as antioxidants. This study aimed to compare the effectiveness of domestic antiprotozoal drugs and a newly developed drug containing plant components against protozoosis in turkeys. The developed combined drug, which includes the antiprotozoal substances metronidazole and furazolidone, as well as the plant components garlic powder (*Allium sativum*) and tansy (*Tanacetum vulgare*), demonstrated a rapid and sustained reduction in mean infection intensity in turkeys, particularly on days 7–14 of treatment. The combined drug's effectiveness exceeded that of Nifulin, evidenced by a significant reduction in pathogen numbers and complete *Blastocystis* spp. elimination by day 14. By day 7, the intensity of the infection decreased by more than 78%. By day 14, the infection was completely eradicated (prevalence — 0%, effectiveness — 100%). When Nifulin was used, however, 21.4% of turkeys remained infected. The drug also provided higher effectiveness at all stages of histomoniasis treatment. On day 14, only 7.1% of turkeys were infected, compared to 14.3% in the Nifulin group. The respective effectiveness was 92.9% and 78.6%.

Keywords: antiprotozoal drugs, metronidazole, furazolidone, *Allium sativum*, *Tanacetum vulgare*

Introduction. The gastrointestinal tract is the body's largest exposed surface and is constantly exposed to various potentially harmful substances. It acts as a selective barrier between poultry tissues and their internal environment (Yegani and Korver, 2008).

Gastrointestinal parasites are a major problem in poultry farming because they cause significant economic losses due to reduced productivity, poor feed conversion, poor weight gain, reduced egg production, loss of appetite, diarrhea, intestinal obstruction, exhaustion, anemia, paralysis, plumage disorders, and even death (Jegede et al., 2015; Divyamery et al., 2016; Balarabe et al., 2017).

The most common etiological agents affecting the gastrointestinal tract of farm poultry are helminths (e.g., nematodes, cestodes, and trematodes) and protozoa. These agents can cause significant disturbances in physiological functions and reduce the overall productivity of the flock (Bayzid et al., 2023; Bogach et al., 2024).

Histomonas meleagridis is a protozoan parasite that primarily affects turkeys and chickens, causing a disease known as histomoniasis. The severity of the disease varies by host species. In turkeys, it often leads to high mortality, whereas in chickens, mortality is usually much lower (Mitra et al., 2018).

According to Hauck and Hafez (2013), mortality in turkeys can reach 100%, as demonstrated in several experimental studies.

The pathogenesis of histomoniasis begins with the parasite colonizing the cecum, which leads to severe inflammation and necrosis. After destroying intestinal tissue, the parasite can enter blood vessels and reach the liver via the portal vein. Consequently, areas of inflammation and destruction can occur in the liver. In the final stage, the disease becomes systemic, and the parasite spreads to various organs of the host (Grabensteiner et al., 2006).

Among drugs with antibiotic and antiprotozoal activity, nitroimidazoles were the only substances that were completely effective against *H. meleagridis*. During the studies, nine drugs were tested both *in vitro* and *in vivo* for their activity against *H. meleagridis*. Nitroimidazoles — dimethridazole, metronidazole, ornidazole, and tinidazole — inhibited parasite growth *in vitro* at concentrations of 10 μg/ml and above. In contrast, paromomycin sulfate and carbadox showed weak efficacy only at high concentrations. Quinolinol, mebendazole, diloxanide furoate, and albendazole did not demonstrate proven efficacy *in vitro* (Hu and McDougald, 2004; Hauck, Lotfi and Hafez, 2010).

For decades, histomoniasis could be effectively controlled with potent preventive and treatment drugs; however, these chemicals were subsequently withdrawn from use in Europe, the United States, and other countries due to consumer protection concerns (CEC, 1995; McDougald, 2005; McDougald et al., 2020).

Effective chemotherapeutic agents, such as nifursol and nitroimidazoles, are no longer permitted in the United States or the European Union. Additionally, the European Union banned arsenic compounds (Baynes et al., 2016).

The treatment of experimentally infected turkeys with *H. meleagridis* using paromomycin at doses of 400 mg/kg or 200 mg/kg in feed or 420 mg/l in water significantly reduced mortality and decreased the severity of cecal and liver lesions (Bleyen et al., 2009).

Blastocystis has been found in many animal species, including mammals, birds, and amphibians (Parkar et al., 2010; Bohach et al., 2023; Bogach, Paliy, and Bohach, 2023).

There is still debate about the advisability of treatment due to limited knowledge about this parasite, as the infection may be opportunistic in nature (Biedermann et al., 2002).

The discontinuation of effective chemotherapeutic drugs in recent decades due to concerns about consumer safety has led to frequent disease outbreaks, threatening the welfare of poultry and causing significant economic losses to the poultry industry. Recent studies have examined various approaches to combating the disease, including alternative chemotherapeutic agents and plant-derived compounds (Liebhart et al., 2017).

In many countries, government agencies regulate the use of drugs for poultry raised for food to eliminate or minimize side effects and ensure consumer protection (FDA, 2025).

Following the European Union's ban on growth-promoting antibiotics, research on methods to improve intestinal health has intensified. Many researchers have described the positive effects of plant compounds on poultry health, and the practical application of these compounds shows promise in poultry farming. Research has proven that adding plant-based feed additives protects birds from environmental threats that lead to intestinal barrier dysfunction. Phytogenic feed additives can improve the overall structure of the intestinal mucosa and the intestinal barrier's function at the molecular level (Latek et al., 2022; Bozkurt and Tüzün, 2020).

Alternative strategies for controlling protozoan infections are being developed to reduce the use of veterinary drugs. Examples of these alternative therapeutic options include natural treatments such as prebiotics and probiotics, plant and fungal extracts, and essential oils. While natural compounds typically do not directly combat parasites, they affect the microflora of the gastrointestinal tract and strengthen the immune system (Abd El-Hack et al., 2022).

Using phytogenic substances in turkeys' diets is promising because they are effective and can act as antioxidants (Bozkurt and Tüzün, 2020).

Essential oils are considered promising preventive and therapeutic agents against a number of flagellate parasites of poultry. In particular, the antiprotozoal activity of essential oils obtained from fresh leaves of *Cinnamomum aromaticum*, pericarp of *Citrus limon*, and bulbs of *Allium sativum* was studied. *In vitro* experiments have established their effect on *Tetratrichomonas gallinarum* and *H. meleagridis*. The results of the study indicate that the effective concentrations of essential oils required to inhibit *T. gallinarum* and *H. meleagridis* differ significantly (Zenner et al., 2003).

The use of herbal preparation containing extracts of cinnamon, garlic, lemon, and rosemary reduced the mortality of turkeys infected with *H. meleagridis* to 20%, while in the control infected group it was 50% (Hafez and Hauck, 2006).

In the context of modern research on natural antiprotozoal agents, 43 plant substances and extracts (aqueous, ethanolic, and heptanic) from 18 types of food and feed industry organic waste were studied. Their activity was tested *in vitro* against *H. meleagridis*, *T. gallinarum*, and *Blastocystis* spp., with the highest efficacy shown by ethanol extracts of thyme, serenoa, grape seeds, and pumpkin. These results indicate the potential of these extracts for preventing and treating parasitic infections in poultry (Grabensteiner et al., 2008).

Despite *Blastocystis*' widespread distribution as an intestinal parasite, the issue of its pathogenicity and effective treatment remains controversial. Metronidazole is the most common therapy, but cases of ineffectiveness and drug resistance have been reported. Paromomycin and trimethoprim-sulfamethoxazole are being considered as alternative treatments (Roberts et al., 2014).

The study **aimed** to compare the effectiveness of domestic antiprotozoal drugs and a developed drug with herbal ingredients for treating protozoosis in turkeys.

Materials and methods. Studies were conducted in the vivarium of the Odesa Research Station of the National Scientific Center 'Institute of Experimental and Clinical Veterinary Medicine' to determine the therapeutic effectiveness of antiprotozoal drugs for mixed blastocystosis and histomoniasis in turkeys. The drugs' effectiveness was evaluated using forty-two 45-day-old 'Big-6' breed turkeys that were spontaneously infected with *Blastocystis* spp. and *H. meleagridis*. Two experimental and control groups were formed from these turkeys (n = 14).

The research was conducted following the recommendations of the 'European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes' (CE, 1986) and Council Directive 2010/63/EU (CEC, 2010), and under Art. 26 of the Law of Ukraine No. 3447-IV of 21.02.2006 'About protection of animals from cruel treatment' (VRU, 2006) and basic bioethical principles (Simmonds, 2017). Under the current procedure, the research program was reviewed and approved by the Bioethics Committee of the National Scientific Center 'Institute of Experimental and Clinical Veterinary Medicine' (Kharkiv, Ukraine).

A microscopic examination was performed to detect *Blastocystis* spp. and *H. meleagridis* in turkeys. Fresh fecal samples were applied to clean, degreased microscope slides and prepared as native smears. The smears were fixed with ethanol for five to ten minutes and stained using the Romanowski–Giemsa method. Then, the slides were examined under a microscope at

630× magnification (90× objective, 7× eyepiece). In the stained smears, the nuclei and flagella of the histomonads appeared red while the cytoplasm appeared blue, allowing for clear visualization of the pathogen.

45-day-old turkeys from the first experimental group were administered an antiprotozoal drug complex (ORS NSC 'IECVM', experimental sample) in powder form at a dose of 2 g/kg of feed for seven days for therapeutic purposes. The turkeys in the second experimental group were given Nifulin (powder) (OLKAR) at a dose of 1 g/kg of feed for seven days for therapeutic purposes. The turkeys in the control group were infected and did not receive any drugs. The dynamics of turkey infection with blastocysts and histomonads, as well as the effectiveness of the drugs, were determined on the third, seventh, and fourteenth days.

The intensity of *Blastocystis* spp. infection was assessed based on the number of parasites observed in a microscope field of view and the presence of clinical manifestations. Low intensity was defined as fewer than five parasites in a field of view and a subclinical course with minor symptoms. Moderate intensity was defined as five to ten parasites and moderately pronounced symptoms, including digestive disorders. High intensity was defined as more than ten parasites and symptoms such as diarrhea, weight loss, and exhaustion, which pose a risk of death to the bird (Tan, 2008).

The intensity of *H. meleagridis* infection was determined using a scale that approximates the number of trophozoites in a microscope field of view: low (1–2 trophozoites; asymptomatic or mild symptoms); moderate (3–10 trophozoites; diarrhea, depression); and high (> 10 trophozoites; pronounced clinical signs, risk of bird death) (McDougald, 2005; Clark and Kimminau, 2017).

A statistical analysis of the data was performed using a free trial version of Minitab 19 software from Minitab, Inc. The arithmetic mean (M) and the standard error of the mean (m) were calculated. The probability of a difference between the means of two series of variations was assessed using the reliability criterion (td), Student's t distribution tables, and the nonparametric Van der Waerden method.

Results. A complex drug containing the antiprotozoal substances metronidazole and furazolidone, as well as the plant components garlic powder (*Allium sativum*) and tansy (*Tanacetum vulgare*), has developed at the Laboratory of Epizootology, Parasitology, Animal Disease Monitoring, and Providing of the Odesa Research Station of the National Scientific Center 'Institute of Experimental and Clinical Veterinary Medicine'. This drug is intended to treat protozoal diseases in poultry, particularly cryptosporidiosis, histomoniasis, eimeriosis, tetratrichomoniasis, and blastocystosis.

Due to the ban on traditional drugs for the treatment of histomoniasis, there is growing interest in alternative remedies. Carvacrol, cassia oil, a mixture of thyme and rosemary essential oils, and *Quillaja saponaria* saponin exhibit pronounced antiprotozoal activity against *H. meleagridis* (minimal inhibitory concentration (MIC) 0.25–0.50 μg/ml) and various isolates of *Blastocystis* spp. (Grabensteiner, Arshad, and Hess, 2007).

Before treatment, the intensity of *Blastocystis* spp. infection in 45-day-old turkeys ranged from 7.2 ± 0.1 to 7.6 ± 0.2 cysts per field of view (FOV), while the intensity of *H. meleagridis* infection ranged from 8.9 ± 0.2 to 9.1 ± 0.2 trophozoites per FOV (Table 1).

Table 1 — Intensity of *Blastocystis* spp. and *H. meleagridis* infection during treatment of 45-day-old turkeys (n = 14, M ± m)

Day	Pathogen	Intensity, number of pathogens in the field of view of the microscope		
		Groups of turkeys treated with		Control
		Developed drug	Nifulin	
Before treatment	*Blastocystis* spp.	7.2 ± 0.1	7.6 ± 0.2	7.6 ± 0.1
	H. meleagridis	9.1 ± 0.2	8.9 ± 0.2	9.0 ± 0.1
3	*Blastocystis* spp.	6.1 ± 0.1***	7.2 ± 0.1*	7.9 ± 0.2
	H. meleagridis	8.2 ± 0.1***	8.1 ± 0.2*	9.1 ± 0.1
7	*Blastocystis* spp.	–	5.1 ± 0.1***	8.2 ± 0.1
	H. meleagridis	1.1 ± 0.1***	3.9 ± 0.1***	9.8 ± 0.2
14	*Blastocystis* spp.	–	2.1 ± 0.1***	8.5 ± 0.1
	H. meleagridis	–	2.0 ± 0.1***	10.1 ± 0.1

Note:s * — $p < 0.05$, *** — $p < 0.001$ — compared to day before treatment.

Three days after the administration of the complex drug, the intensity of *Blastocystis* spp. infection decreased significantly ($p < 0.001$) by 15.3%, while the intensity of *H. meleagridis* infection decreased by 9.9%. By the seventh day, *Blastocystis* cysts were undetectable in fecal samples, while the number of *H. meleagridis* trophozoites was 1.1 ± 0.1 in the FOV ($p < 0.001$), indicating a low level of infection (fewer than five parasites) and a subclinical course. By day 14 after treatment, neither *Blastocystis* spp. cysts nor *H. meleagridis* trophozoites were detected in the experimental turkeys, indicating the pathogens were eliminated by the drug.

After the use of Nifulin, on the third day, a significant ($p < 0.05$) decrease in the number of *Blastocystis* spp. cysts by 5.3% and *H. meleagridis* trophozoites by 9.0% ($p < 0.05$) was observed. On the 7th day, the intensity of infection decreased significantly ($p < 0.001$) by 32.9% for *Blastocystis* spp. and by 56.2% ($p < 0.001$) for *H. meleagridis*. After 14 days, the number of *Blastocystis* spp. decreased significantly ($p < 0.001$) by 72.4% to 2.1 ± 0.1 cysts in FOV, indicating a low level of invasion (less than 5 parasites, subclinical course). During this period, the intensity of *H. meleagridis* infection reliably decreased ($p < 0.001$) by 77.5% to 2.0 ± 0.1 trophozoites in FOV. Meanwhile, turkeys exhibited mild clinical signs, including slight depression, moderate decrease in appetite, and slight growth retardation.

In the control group of untreated turkeys, the intensity of *Blastocystis* spp. infection gradually increased from an average of 7.6 ± 0.1 to 8.5 ± 0.1 cysts in the FOV. This level of infection corresponds to the average range of 5–10 parasites in FOV and was clinically manifested by moderate symptoms, including digestive disorders. Concurrently, the number of *H. meleagridis* trophozoites increased from 9.0 ± 0.1 to 10.1 ± 0.1 in FOV, indicating a high level of infection (more than 10 trophozoites in FOV). The turkeys exhibited pronounced clinical signs, including diarrhea, weakness, and weight loss.

Thus, 14 days after treatment, the degree of reduction in infection intensity for the developed complex drug was 100% for both *Blastocystis* spp. and *H. meleagridis*, indicating the complete elimination of the pathogens from turkeys' intestines. In the group that received Nifulin, the reduction in infection intensity was 72.4% against *Blastocystis* spp. and 77.5% against *H. meleagridis*, indicating noticeable but incomplete suppression of the invasion.

According to Yakoob et al. (2011), metronidazole at a concentration of 0.01 mg/mL reduced the number of *B. hominis* cysts in the microscope field of view to approximately 12 cysts, and at 0.1 mg/ml, the number decreased to approximately 1–2 cysts. Garlic had an even more pronounced effect, decreasing the number of cysts in the field of view from 30 to 1 at 0.01 mg/ml and practically eliminating them (0–0.1 cysts) at 0.1 mg/ml. White cumin and black pepper reduced the level of infection to approximately 10 cysts in FOV at 0.1 mg/ml. Ginger reduced the number of *B. hominis* cysts to approximately 13 in FOV, indicating moderate activity.

After the birds in the first experimental group were treated on day 3, *Blastocystis* spp. cysts were detected in nine turkeys and *H. meleagridis* trophozoites in eight turkeys (Table 2).

Table 2 — Effectiveness of drugs against blastocystosis and histomoniasis in 45-day-old turkeys (n = 14)

Groups	Pathogen	Prevalence, %			Effectiveness of the drug, %		
		Day					
		3	7	14	3	7	14
I experimental	*Blastocystis* spp.	64.3	–	–	35.7	100.0	100.0
	H. meleagridis	57.1	14.3	–	42.9	85.7	100.0
II experimental	*Blastocystis* spp.	78.6	42.9	21.4	21.4	57.1	78.6
	H. meleagridis	71.4	35.7	14.3	28.6	64.3	78.6
Control	*Blastocystis* spp.	100.0	100.0	100.0	–	–	–
	H. meleagridis	100.0	100.0	100.0	–	–	–

By day 7, *Blastocystis* spp. were no longer present in the experimental turkeys, suggesting that the drug was 100% effective against this pathogen. Meanwhile, the drug was 85.7% effective against *H. meleagridis*. By the 14th day of the study, the drug was 100% effective against both pathogens, indicating complete eradication of these protozoan infections in turkeys.

In the second experimental group, *Blastocystis* spp. cysts were detected in 11 turkeys, and *H. meleagridis* trophozoites were detected in 10 turkeys after the use of Nifulin on the third day. By the seventh day, the prevalence of both pathogens had decreased by 35.7%, and the drug's effectiveness was 57.1% for *Blastocystis* spp. and 64.3% for *H. meleagridis*. By the 14th day, *Blastocystis* spp. was detected in three birds and *H. meleagridis* in two, indicating effectiveness rates of 64.3% and 78.6%, respectively. Throughout the study period, the control group of turkeys constantly excreted *Blastocystis* spp. cysts and *H. meleagridis* trophozoites.

In vitro studies have shown that extracts from Thai medicinal plants traditionally used to treat diarrhea exhibit varying degrees of activity against *B. hominis*. Dichloromethane and methanol extracts from *Brucea javanica* seeds, as well as methanol extract from *Quercus infectoria* nut branches, demonstrated the highest effect at a dose of 2,000 μg/ml. These extracts destroyed 82%, 75%, and 67% of isolates, respectively, and inhibited their growth by 94%, 100%, and 76%, respectively. By comparison, metronidazole provided 97% eradication and complete inhibition of *Blastocystis* at concentrations of 1.25–20 μg/ml (Sawangjaroen and Sawangjaroen, 2005).

We evaluated the efficacy of benzimidazole derivatives (albendazole and fenbendazole) in treating and preventing histomoniasis in turkeys. Metronidazole produced greater body weight gain and reduced cecum and liver damage compared to the control group. However, albendazole (100 mg/kg) and fenbendazole (10 mg/kg) were ineffective as therapeutic agents when

administered orally for five days, as confirmed during the evaluation of their prophylactic effect (Hegngi et al., 1999). One hour after Ag NPs were applied, the number of *B. hominis* cysts decreased by 20.7%. With metronidazole saturated with Ag NPs, the decrease was 28.2%, and with metronidazole alone, the decrease was 18.9%. Three hours later, the decrease was 71.7%, 79.7%, and 62.7%, respectively, indicating the higher efficacy of combining Ag NPs with metronidazole (Younis et al., 2020).

Conclusions. The developed combined drug demonstrated higher therapeutic efficacy against a mixed infection of turkeys with *Blastocystis* spp. and *H. meleagridis* than Nifulin did. By the 7th day of treatment, there was a 78% reduction in *Blastocystis* spp. infection intensity, and by the 14th day, the pathogen was completely eliminated (prevalence — 0%, effectiveness — 100%). The drug decreased the level of histomoniasis infection more significantly, reducing the number of infected turkeys to 7.1% (effectiveness — 92.9%), which exceeds the effectiveness of Nifulin (effectiveness — 78.6%). This high effectiveness is due to the combination of chemotherapeutic components and phytotherapy, which boosts immunity and enhances the overall effect.

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STUDY OF THE BIOCHEMICAL PROPERTIES OF *BORDETELLA BRONCHISEPTICA* CLINICAL ISOLATES

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Summary. During the first half of 2025, we isolated five isolates of *Bordetella bronchiseptica* from dogs with bordetellosis. These isolates exhibited low enzymatic activity, which is characteristic of *B. bronchiseptica*. Specifically, they did not produce indole, liquefy gelatin, ferment sugars, or produce the enzyme tyrosinase. However, they produced the enzyme urease and exhibited catalase and oxidase activity. Additionally, they grew on Simmons citrate agar and reduced nitrates to nitrites. Notably, they did not produce hydrogen sulfide on trisaccharide agar with the addition of iron. Our improved method for identifying and differentiating *B. bronchiseptica* isolates enabled us to significantly simplify and accelerate the isolation of pure *Bordetella* cultures by 2–3 days. Identification of clinical isolates of *B. bronchiseptica* took 3–4 days

Keywords: Bordetellosis, dogs, identifying, differentiating, pure culture

Introduction. There are many modern methods for detecting, characterizing, and identifying microorganisms. Some methods rely on phenotypic biochemical characteristics, while others use genotypic identification. The biochemical characteristics of bacteria offer numerous features that facilitate the classification and identification of microorganisms (Korniienko et al., 2008; Gerlach et al., 2001; Gueirard et al., 1995; Harvill et al., 2000).

The preferred and generally accepted approach for determining the genus and species of a bacterium is to analyze the nutritional and metabolic capabilities of a bacterial isolate. This approach allows for the quick and accurate identification of microorganisms without the need for costly equipment and materials (Korniienko et al., 2008; Henderson and Nataro, 2001; Ellis, 2015; Hadzevych, 2024).

Studying enzymes in bacteria is also of great practical importance. Determining microbial enzyme activity allows one to differentiate between bacterial species and identify the nature of a pathogen. Additionally, microbial enzyme activity determines the pathogenesis and clinical presentation of infectious diseases.

Studying biochemical activity in bacterial isolates is crucial for microbiological laboratories and clinical practice because the results guide treatment choices and help prevent and control disease spread among animals and humans. For example, cases of salmonellosis require rapid and precise identification of the pathogen to trace the infection source and enable healthcare providers to prescribe the right treatment quickly. Accurately identifying the genus and species of the pathogen during an outbreak is also vital so that an appropriate response can be implemented promptly to control the epizootic (epidemic) (Gueirard et al., 1995; Harvill et al., 2000; Kameyama et al., 2022; Milanko, Kholodylo and Dushkyn, 1995).

There are currently many reports about the difficulty of diagnosing bordetellosis in animals. The etiological agent of the disease is the bacterium *B. bronchiseptica*. This Gram-negative bacterium colonizes the respiratory tract of mammals. Bordetellosis is a highly contagious infectious disease in animals that often progresses chronically and asymptomatically, which greatly complicates diagnosis, prevention, and treatment (Korniienko et al., 2008; Harvill et al., 2000; Henderson and Nataro, 2001; Hadzevych, 2024). Laboratory diagnosis of bordetellosis is also complicated by the fact that the disease is often uncontrollable and spreads rapidly through asymptomatic carrier animals. Therefore, it is urgent to search for and develop test systems suitable for the early detection and identification of the pathogen and its phase states. The main conditions for successfully eliminating outbreaks of bordetellosis in animals are the early isolation of the pathogen and determination of its properties and antibiotic sensitivity; timely initial treatment; and the comprehensive, systematic implementation of remedial measures, including general and specific veterinary and sanitary measures (Korniienko et al., 2008; Milanko, Kholodylo and Dushkyn, 1995; Mylanko and Dushkin, 1996; Milanko, Gerilovich and Dushkin, 1995).

According to the literature, the bacteriological method remains the main tool for diagnosing bordetellosis in animals. This method includes isolating a pure culture of the bacterium and determining its biochemical activity. At the same time, many scientists claim that the study takes 6–9 days (MHU, 2005). The duration of the study is associated with the pathogen's slow growth, the untimely and incomplete examination of animals with prolonged coughs, contamination of the test material by other microorganisms, the use of antibacterial drugs before the study's start, violations of the rules for collecting and transporting material, and unsuccessful or imperfect formulations of diagnostically selective media. In particular, there is an insufficient selection of selective and nutrient components (Harvill et al., 2000; MHU, 2007; Gadzevich, 2024; Moore, Rendall and Millar, 2021a, 2021b; Inatsuka, Julio and Cotter, 2005; Jacob-Dubuisson et al., 2000; Chambers

et al., 2019; Schulz et al., 2014; Taha-Abdelaziz et al., 2016; Miguelena Chamorro et al.; 2023, Kadlec and Schwarz, 2018; Clements, McGrath and McAllister, 2018).

Thus, diagnosing bordetellosis and studying the properties of *B. bronchiseptica* require careful research and study. Resolving these issues will enable the integration of modern tools into veterinary practices, enhance diagnostic methodologies, and provide valuable insights into effective outbreak prevention strategies. Our study aimed to investigate the biochemical properties of clinical isolates of *B. bronchiseptica* that we collected from sick dogs in 2025. We **aimed** to determine the terms for studying the biochemical activity of *Bordetella*, identify weaknesses in studying bacterial enzymes, and find solutions to eliminate them. Based on the results, we planned further work to improve the diagnosis of bordetellosis in animals.

Materials and methods. Clinical isolates of *B. bronchiseptica* were isolated from dogs with or suspected of having bordetellosis in accordance with the methodological guidelines for the microbiological diagnosis of whooping cough and paracough. These guidelines were approved by Order No. 169 of the Ministry of Health of Ukraine on April 15, 2005, in our modification (Hadzevych, 2024; MHU, 2005; Woods et al., 2019; Bemis, Shek and Clifford, 2003). The process of isolating *B. bronchiseptica* clinical isolates from nasopharyngeal secretions consisted of two stages. The first stage included selecting and culturing the biological material (nasopharyngeal secretions) on a nutrient medium. Based on positive results from previous studies, we used a CCA (casein-charcoal agar) medium supplemented with 5% sheep blood and the selective component cephalexin (4 mg/100 ml), adding the antibiotic to the medium. Samples of nasopharyngeal secretions for *Bordetella* isolation were collected using a sterile, disposable probe swab from the mucous membranes of the tonsillar and peripharyngeal areas. In parallel, the biological material was inoculated onto other nutrient media (MPA, MPB, blood agar, Endo, MacConkey, Sabouraud, etc.) to isolate and identify other associated microorganisms (Hadzevych, 2024; MHU, 2005). Inoculations were performed to obtain separate colonies (MHU, 2005). The inoculations were placed in a thermostat at a temperature of 35.5 ± 0.5°C for 48 h. The growth of microorganisms on the CCA medium was assessed daily. The morphology of the colonies was examined using an MBS-9 binocular magnifying microscope with a long focal length (Korniienko et al., 2008; Harvill et al., 2000; Henderson and Nataro, 2001; MHU, 2005; Bemis, 1992).

Samples from sick animals were collected early in the disease process, no later than three weeks after the onset of symptoms, because the viability of the pathogen drops significantly later on. It was crucial to ensure that the animals were not treated with antibiotics before the biological material was collected. To obtain objective results for isolating clinical isolates of *B. bronchiseptica*, eight samples of nasopharyngeal secretions were collected from each dog's tonsillar and peripharyngeal mucous membranes using a sterile, disposable probe-swab for each sample. Samples of nasopharyngeal secretions from the same sick dogs were collected twice in a row during repeated examinations at 24-hour intervals. The media and individual nutrient components were produced by HiMedia Laboratories Pvt. Ltd. (India) and Farmaktiv LLC (Ukraine) (Korniienko et al., 2008; Gerlach et al., 2001; MHU, 2005, 2007).

The second stage was to obtain a pure culture of the *B. bronchiseptica* clinical isolate. To this end, cultures on nutrient media were examined. Based on the appearance of the colonies (size, color, and shape), certain rapid tests, and light microscopy after staining with aniline dyes, the type of microorganisms and their significance in a particular case were determined (Ellis, 2015; Hadzevych, 2024; MHU, 2005, 2007). Selected colonies were screened on nutrient media to accumulate a pure culture of microorganisms. If a sufficient number of colonies of only one species grew, identification and determination of antibiotic sensitivity were carried out without the accumulation stage.

The identification of the isolated microorganisms, including the determination of their biochemical, serological, and antigenic properties, was performed using a fixed set of substrates, diagnostic selective media, and sera. These substrates included carbohydrates, amino acids, polyhydric alcohols, and other complex compounds. The results were used to determine the genus and species of the microorganism (Ellis, 2015; Hadzevych, 2024; Kameyama et al., 2022; MHU, 2005). To study the biochemical properties of the microorganisms, we used selective and diagnostic selective media, as well as individual components, which were manufactured by HiMedia Laboratories Pvt. Ltd. (India) and Farmaktiv LLC (Ukraine). These included Giss medium with sugars and polyhydric alcohols, acetate agar, nitrate agar to determine nitrate reduction to nitrite, a medium to determine gelatinase, Piz medium, and Simmons citrate agar. The reference strains used were *B. bronchiseptica* B-C2, *B. bronchiseptica* No. K16, and *B. bronchiseptica* No. K17. These strains were stored in the Microorganism Strain Museum of the National Scientific Center 'Institute of Experimental and Clinical Veterinary Medicine' (Kharkiv, Ukraine).

To determine the catalytic activity of clinical isolates of *Bordetella bronchiseptica*, a microbial sample was placed on a microscope slide with a loop, and a drop of 3% hydrogen peroxide solution was immediately added to it. The release of oxygen bubbles indicated the presence of catalase in the microbes.

To detect urease activity, the Zaks method was used, which is based on the ability of microorganisms with the urease enzyme to break down urea into ammonia. This causes a change in the pH of the medium, which is detected by a change in the color of the indicator. After incubation, the results were recorded. If there was no change in color, the tubes were left at room temperature,

and the results were recorded the next day. If the microorganism has the urease enzyme, the urea breaks down into ammonia, which leads to alkalization of the medium and a color change from yellow to crimson (Hadzevych, 2024; Mylanko and Dushkin, 1996).

The ability to use citrate as the sole carbon source was determined using Simmons medium. The test culture was inoculated onto sloping Simmons medium and incubated at 36 ± 1°C for one day. *B. bronchiseptica*, a citrate-utilizing bacterium, grows well, alkalizes the medium, and causes it to turn blue. To determine pigment formation, the culture was inoculated onto simple nutrient agar containing 0.1% tyrosine and incubated at 36 ± 1°C for 24 h. When tyrosine is broken down, the medium turns yellow-brown.

Results and discussion: Based on bacteriological studies of nasopharyngeal secretion samples collected in the first half of 2025, five clinical isolates of *B. bronchiseptica* were isolated from five dogs (n = 5) with bordetellosis. The disease progressed acutely and classically in all dogs. The animals exhibited decreased or lost appetite, lethargy, and mucopurulent (Fig. 1) or purulent (Fig. 2) nasal discharge. They also displayed conjunctivitis (Fig. 3), enlarged submandibular lymph nodes, and an increased body temperature of 40–41°C. The dogs experienced catarrhal and subsequently catarrhal-purulent inflammation of the upper respiratory tract, as well as shortness of breath, frequent sneezing, and a painful, suffocating cough. The cough intensified when the dogs stood up or moved, changing from wet to dry with severe attacks of suffocation and vomiting. As pathogenic microflora accumulated, pulmonary emphysema, pneumonia, exhaustion, and death were observed (Fig. 4).

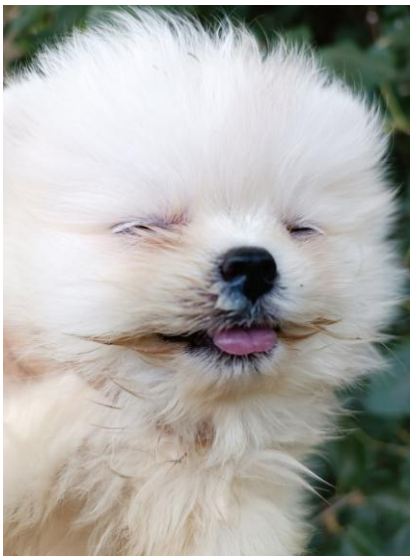

Figure 1. Conjunctivitis and mucopurulent exudate from the nasal passages in a puppy with bordetellosis.

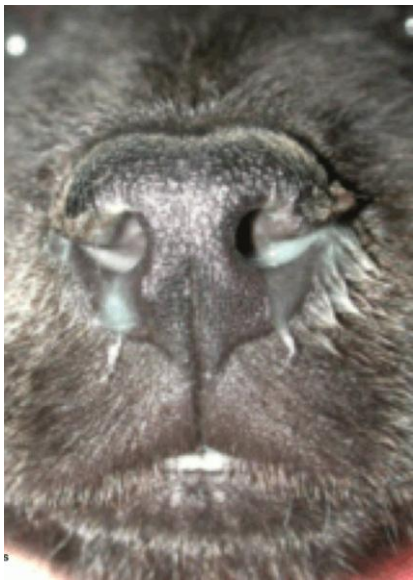

Figure 2. Purulent exudate from the nasal passages in a puppy with bordetellosis.

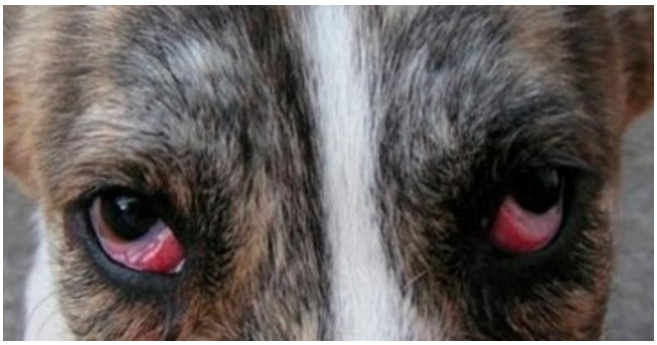

Figure 3. Conjunctivitis in a puppy with bordetellosis.

Figure 4. Signs of exhaustion in a dead animal with pale and dull visible mucous membranes and dry, inelastic skin.

Similar clinical pictures of acute *Bordetella* infection in dogs have been observed by Chambers et al. (2019) and Miguelena Chamorro et al. (2023) among other researchers. However, some reports indicate that bordetellosis in dogs is more often manifested by sneezing and nasal discharge and other scientists also indicate that, in most cases, bordetellosis is mild and resolves within two to three weeks (Miguelena Chamorro et al., 2023; Kadlec and Schwarz, 2018). However, complications can develop, which can lead to pneumonia and death. In our opinion, risk factors for developing complications include an impaired immune system and chronic respiratory diseases, such as bronchitis, ciliary dyskinesia, and tracheal collapse. This view is consistent with studies by Clements, McGrath and McAllister (2018), Woods et al. (2019), and other scientists. These studies also indicate that the nature and intensity of the infection depend solely on the pathogen's aggressive properties, the animal's immune status, and the presence of accompanying microflora.

Based on our study of the phenotypic characteristics of the *B. bronchiseptica* clinical isolates selected, we established that all five isolates formed colonies on a dense CCA medium within 24–48 h of cultivation at 35.5 ± 0.5°C during primary isolation. Subsequent recultivations on CCA medium reduced the time required for colony formation to 18–24 h. Similar growth dynamics and colony formation times for *B. bronchiseptica* were observed by Schulz et al. (2014) and other scientists. They noted that, due to *Bordetella*'s adaptation to artificial cultivation conditions, the growth rate of microorganisms and colony formation accelerated. The *Bordetella* isolates grew on dense media in the form of polymorphic colonies that varied greatly in color, shape, size, and consistency.

We more often recorded the formation of colonies of three types of subcultures of isolated clinical isolates of *B. bronchiseptica*:

— convex, moist, smooth, and shiny with even edges. They were gray in color, sometimes with a blue, pearl, metallic, yellowish, or whitish tint. They were 0.5–2 mm in diameter. These colonies had a soft, oily consistency and could be easily removed with a loop;

— flat colonies or those with a raised (or conversely, depressed) center were oily and gray with a blue, yellowish, or whitish tint;

— in the form of a diffuse, oily, gray mass (diffuse growth) with different shades and often without clear boundaries.

Morphologically, three phases of colony development were distinguished. Phase I corresponded to the S-shape, colonies were round, convex, moist, microorganisms were monomorphic and small. Recently isolated and young cultures were predominantly in Phase I of colony development, as determined by morphological characteristics (Fig. 5). Phases II (SR-form) and III (R-form) were transitional, with changes in bacterial morphology. In the second phase, rods of various sizes were predominantly found, while in the third phase, coccoid formations of various sizes were found. Colonies in phases II and III were slightly larger in size, with a diameter of 1–2 mm. The colonies were flat, slimy, with a tendency to merge, and had predominantly uneven contours and different segments (Figs. 6 and 7).

As shown in Fig. 6, growth of *B. bronchiseptica* culture on blood agar was observed after 24 h of incubation at a temperature of 35.5 ± 0.5°C in the form of a diffuse oily gray mass (confluent growth) with different shades and uneven edges. Fig. 7 shows an increase in the size of the growth zone of *B. bronchiseptica* culture on blood agar after 48 h of incubation due to diffuse growth of *Bordetella* on the surface of the medium. Fig. 8 shows the growth of *B. bronchiseptica* culture in the form of a continuous film on the surface of semi-liquid (0.4%) nutrient agar after 48 h of incubation at a temperature of 35.5 ± 0.5°C.

Based on our analysis of the literature, we found that Miguelena Chamorro et al. (2023), Kadlec and Schwarz (2018) and other researchers distinguish three phases of *B. bronchiseptica* colony development based on morphology: I — the virulent phase, which corresponds to the S-form; II — the weakly virulent phase (SR-form); III — the avirulent phase (R-form). Foreign researchers have determined that phase I, the virulent phase, is cultivated on Bordet-Gengou agar during primary isolation. The resulting colonies are round, convex, and moist. Prolonged cultivation results in antigenic modulation and transformation of the microorganism into phases II and III. Phase II is weakly virulent, and phase III is avirulent. The colonies are flat and mucous in morphology with a tendency to merge. They have predominantly uneven contours and different shades. This phenomenon is reversible, and scientists have shown that a return to phase I is possible even after 15 passages. This conclusion is fully consistent with the results of our research. Our research shows that cultures isolated after 18–24 h of incubation at a temperature of 35.5 ± 0.5°C more frequently formed small, smooth, shiny, translucent colonies with even edges and a convex surface. These colonies ranged from 0.5 to 2.0 mm in diameter and turned grayish-white after 48–72 h. The diversity of *B. bronchiseptica* colonies makes it harder to distinguish them from foreign microflora, which extends the time needed to isolate a pure *Bordetella* culture. To shorten this process when suspicious colonies are present on the culture medium, we transferred a pure *Bordetella* culture onto Petri dishes with CCA medium. The medium's surface was divided into several sectors, and each colony was transferred to a different sector. The cultures were then incubated at a temperature of 35.5 ± 0.5°C for 24–48 h. Since extraneous microflora can grow when sample material is cultured on nutrient media, we transferred the maximum number of colonies, on average, at least five.

Based on the results of our analysis of literature data and our own research on the phenotypic characteristics of *B. bronchiseptica* clinical isolates, we can conclude that the typical bacterial cell morphology, color characteristics,

and ability to grow on specific nutrient media — taking into account biochemical substrate degradation — as well as phenotypic colony heterogeneity, are unique morphological, tinctorial, biochemical, and cultural properties of *Bordetella*. These properties can be used to assert the presence of the pathogen with a high degree of probability and as an additional tool for its bacteriological detection.

To speed up the isolation of pure *B. bronchiseptica* cultures, we improved the method of identifying and isolating pure *Bordetella* cultures by setting up separate rapid biochemical tests for preliminary identification. The method consisted of the following steps:

1. Obtaining a sufficient quantity of typical colonies on CCA medium by successively plating individual colonies on sectors.

2. Preparing smears and determining the morphological and tinctorial properties of the culture by Gram staining. *B. bronchiseptica* bacteria are Gram-negative, monomorphic, small, ovoid rods (coccobacilli). They do not form spores and are motile and evenly distributed in the smear or arranged in single groups.

3. Performance of the Zaks test to determine the presence of the urease enzyme. If the microorganism produces urease, the urea will break down into ammonia. This leads to the alkalization of the medium, changing its color from yellow to crimson. *B. bronchiseptica* bacteria produce urease (Fig. 9).

4. Determining the catalytic activity of suspicious colonies. *B. bronchiseptica* is catalase-positive (Fig. 10).

5. Determination of the ability to utilize citrate on Simmons medium. *B. bronchiseptica* is a citrate-utilizing microorganism. They grow well, acidify the medium, and cause it to turn blue (Fig. 11).

Based on the study of the pure culture's morphology, the results of the urease, citrate utilization, tyrosinase, and catalase activity tests (Table 1), we made a preliminary conclusion that the microorganism belongs to the *B. bronchiseptica* species. If no colonies suspicious for *Bordetella* bacteria were detected on the culture medium within five days, the study was terminated, and a final negative result was issued.

As shown in Table 1, all five *B. bronchiseptica* clinical isolates that we obtained from dogs with bordetellosis in 2025 had biochemical properties consistent with those of *B. bronchiseptica*, as determined by rapid biochemical testing. To confirm the isolates belonged to the *B. bronchiseptica* species, we performed PCR diagnostics and studied the enzymatic activity of the clinical isolates in more detail. The *B. bronchiseptica* isolates we obtained from dogs exhibited low enzymatic activity, which is characteristic of this species (Table 2): they did not produce indole, liquefy gelatin, or ferment sugars; they exhibited oxidase activity; they reduced nitrates to nitrites; they grew well on MPA, MacConkey agar, and SS agar; and they did not produce hydrogen sulfide on trisaccharide agar with iron. The low biochemical activity of clinical isolates of *B. bronchiseptica* has also been reported by Clements, McGrath and McAllister (2018), Bemis (1992) and other researchers. They note that *B. bronchiseptica* differs from other *Bordetella* species in its ability to reduce nitrates to nitrites. *Bordetella* utilizes certain organic compounds (succinate, citrate, pyruvate, acetate, fumarate, lactate, oxaloacetate, α-ketoglutarate, or amino acids — proline, glutamate, glutamine, and tyrosine) as its sole source of energy (electrons and protons). They suggest that the tricarboxylic acid cycle (Krebs cycle) in the metabolism of *B. bronchiseptica* is nonfunctional. There is speculation that it may be inactive due to the acquisition of additional biochemical pathways for utilizing alternative nutrient sources, rather than due to a lack of enzymes. When studying the biological properties of isolates, scientists found that *Bordetella* bacteria can be cultivated on conventional and selective media. We obtained similar results when studying the cultural properties of isolates. However, there are many reports that *B. bronchiseptica* bacteria are demanding in terms of cultivation conditions and require special nutrient components, especially during primary isolation.

Our improved method for indicating and differentiating clinical isolates of *B. bronchiseptica* has allowed us to significantly simplify and speed up the process of isolating a pure *Bordetella* culture by 2–3 days. From the time of collecting nasopharyngeal swabs from animals and inoculating the sample on CCA to obtaining a pure culture, it now takes only 24–48 h. We allocate 24 h for morphological and tinctorial assessment of *B. bronchiseptica*, rapid testing, and preliminary species identification, and an additional 24 h for the final confirmation of isolates belonging to *B. bronchiseptica* based on enzymatic activity assessment. Overall, the identification process for *Bordetella* now takes 3–4 days.

The literature and our research results indicate that there is a need to improve bacteriological diagnostic methods for bordetellosis, especially for rapid isolation, typing, and differentiation of the pathogen. Developing high-quality selective media and enhancing biochemical tests play a crucial role in this effort. When designing differential and diagnostic media, it is essential to consider which microorganisms — either primary symbionts or associates of the pathogen — should be separated. Specifically, it is necessary to identify which morphologically and phenotypically similar bacterial genera need to be differentiated from clinical isolates of *B. bronchiseptica* first. We believe that establishing and implementing clear criteria for differentiation, including biochemical markers, would be beneficial. Chambers et al. (2019), Miguelena Chamorro et al. (2023), and other researchers report that the primary associated microorganisms in the upper respiratory tract of animals, which need to be differentiated from *B. bronchiseptica*, include *Pseudomonas* spp., *Corynebacterium* spp., *Proteus* spp., *Haemophilus influenzae*, *H. haemolyticus*, *H. parainfluenzae*, *H. parahaemolyticus*, *M. catarrhalis*, *N. meningitidis*, *N. flavescens*, *Yersinia pseudotuberculosis*, *Morganella coli*, and closely related species such as *Bordetella parapertussis* and *Bordetella pertussis*.

Figure 5. Convex, moist, and smooth 0.5–2 mm colonies of the recently isolated *B. bronchiseptica* clinical culture on blood agar after 24 h of incubation.

Figure 6. Growth of *B. bronchiseptica* on blood agar after 24 h of incubation.

Figure 7. Growth of *B. bronchiseptica* on blood agar after 48 h of incubation.

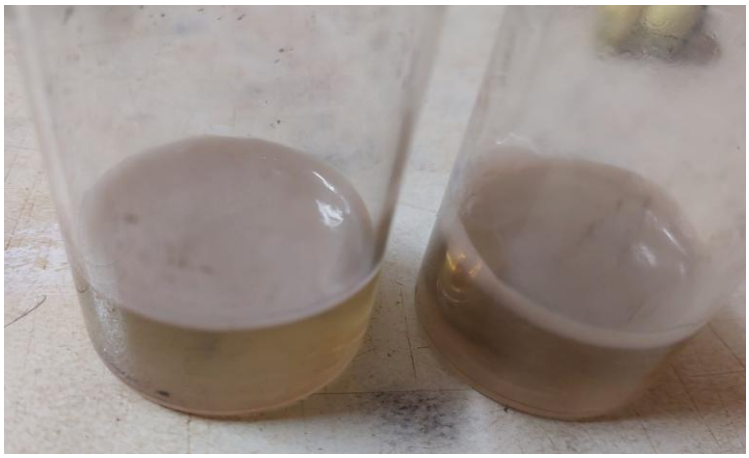

Figure 8. Growth of *B. bronchiseptica* on semi-liquid (0.4%) nutrient agar after 48 h of incubation in the form of a continuous film.

Figure 9. Zaks' tests for determining the urease enzyme. *B. bronchiseptica* has the urease enzyme; we observed the alkalization of the medium and a change in its color from yellow to crimson due to the breakdown of urea into ammonia.

Figure 10. Determination of catalytic activity in clinical isolates of *B. bronchiseptica*. The release of oxygen bubbles indicated the presence of catalase in the microbes.

Figure 11. Determination of the ability to utilize citrate as the sole source of carbon. Citrate-utilizing bacteria *B. bronchiseptica* grew, acidified the Simmons medium, and caused it to turn blue.

Table 1 — Results of biochemical rapid tests for preliminary differential identification of clinical isolates of *B. bronchiseptica*

Designation of microorganisms	Tyrosidase activity	Presence of the urease enzyme	Catalytic activity	Growth on Simmons citrate agar
B. bronchiseptica strain B C2	–	+	+	+
B. bronchiseptica strain K16	–	+	+	+
Isolate 25/1	–	+	+	+
Isolate 25/2	–	+	+	+
Isolate 25/3	–	+	+	+
Isolate 25/4	–	+	+	+
Isolate 25/5	–	+	+	+

Table 2 — Results of biochemical tests for final differential identification of clinical isolates of *B. bronchiseptica*

Designation of microorganisms	Ability to ferment sugars	Ability to liquefy gelatin	Ability to produce indole	Mobility	Oxidase activity	Ability to reduce nitrites to nitrites	Ability to produce hydrogen sulfide on trisaccharide agar with iron
B. bronchiseptica strain B C2	–	–	–	+	+	+	–
B. bronchiseptica strain K16	–	–	–	+	+	+	–
Isolate 25/1	–	–	–	+	+	+	–
Isolate 25/2	–	–	–	+	+	+	–
Isolate 25/3	–	–	–	+	+	+	–
Isolate 25/4	–	–	–	+	+	+	–
Isolate 25/5	–	–	–	+	+	+	–

Conclusions. 1. During the first half of 2025, we isolated five clinical isolates of *B. bronchiseptica* from dogs with bordetellosis. These isolates exhibited low enzymatic activity, which is characteristic of *B. bronchiseptica*. Specifically, they did not produce indole, liquefy gelatin, ferment sugars, or produce the enzyme tyrosinase. However, they did produce the enzyme urease and exhibited catalase and oxidase activity. Additionally, they grew on Simmons citrate agar and reduced nitrates to nitrites. Notably, they did not produce hydrogen sulfide on trisaccharide agar with iron.

2. The characteristics of typical bacterial cell morphology, coloration, and the ability to grow on specific nutrient media, considering the biochemical degradation of various substrates, as well as phenotypic colony heterogeneity, constitute a set of unique morphological, tinctorial, biochemical, and cultural properties of *Bordetella*. These properties can be used to preliminarily identify the microorganism and serve as an additional tool for the bacteriological detection of the pathogen.

3. Our improved method for identifying and differentiating clinical isolates of *B. bronchiseptica* significantly reduces the time required to isolate a pure culture of *Bordetella* by 2–3 days. Identifying clinical isolates of *B. bronchiseptica* took 3–4 days.

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MICROBIOLOGICAL STUDY OF FIELD CULTURES OF *MYCOBACTERIUM* SPP. AS CONTAMINANTS OF MILK AND THE ENVIRONMENT

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Summary. Atypical nonpathogenic nontuberculous mycobacteria are common in the environment and can contaminate livestock facilities, feed, animals, animal products, and manure. These prokaryotes are saprophytes, but they share antigenic similarities with emerging mycobacterial pathogens. Upon contact with animals, they trigger an immune response through complexes of mycobacterial antigens in the host. This can cause errors in immunodiagnostic tests and microbiological assessments of outbreak situations. Microbiological monitoring of livestock products and milk has shown widespread environmental contamination with nonpathogenic, saprophytic, atypical mycobacteria of various species, mainly group IV by Runyon. These are fast-growing, hardy prokaryotes tolerant to a wide range of cultivation conditions and temperatures, with broad adaptability for extracellular enzymatic breakdown of organic macromolecules. The epidemiological concern is that atypical mycobacteria belong to the genus *Mycobacterium* and share antigenic complexes with pathogenic mycobacteria, leading to false-positive allergic reactions and diagnostic confusion in microbiological tests. Biological testing on guinea pigs demonstrated that ubiquitous atypical mycobacteria, common contaminants of milk and manure, do not cause pathological changes in internal organs during necropsy. However, during simultaneous allergy testing, they had significantly stronger reactions to sensitin from atypical mycobacteria. In contrast, responses to tuberculin from pathogenic mycobacteria were weak or absent. Saprophytic mycobacteria are part of the normal, transient microbiota of animals and serve as a barrier in integumentary tissues

Keywords: atypical mycobacteria, ubiquity, nonpathogenicity, manure and milk contamination, false-positive allergic reactions, bioassay, guinea pigs

Introduction. The microbial world of the environment is characterized by endless biodiversity of representatives from all four domains of life, based on the dominance of carbon in metabolic processes that support life and the functioning of genetic information. The most widespread organisms in natural environments, in terms of species diversity and total biomass, are prokaryotic cellular organisms from the domains Bacteria and Archaea. They are phylogenetically the oldest forms of cellular life, tracing back to LUCA (Hotsulia et al., 2021; Kassich et al., 2019; Zazharskyi et al., 2023).

For about four billion years, microbial forms of organic life have carried out the functions of global biogeochemical cycling through enzymatic extracellular dissimilation of complex organic biomolecules. Nonpathogenic mycobacteria are among the natural members of microbial communities that break down organic matter. They actively participate in various biodegradation processes and play an important role in soil formation, working closely with actinomycetes and other organisms of different taxonomic groups. Nonpathogenic mycobacteria make up a large group of prokaryotic species that have adapted to the metabolic conditions of the inanimate environment. During the first two billion years of Earth's formation, they existed solely as saprophytes (Runyon, 1965; Tkachenko et al., 2010; Zazharskyi et al., 2023).

However, as multicellular life forms evolved, interspecies relationships with parasitic potential emerged. This resulted in the development of infectopathology, which is the macroorganism's protective and adaptive response to the invasion of genetically foreign substances into its internal environment. Of the many mycobacterial prokaryotes, only a few species have become pathogenic toward poikilothermic and homeothermic multicellular animals. In the last seven million years, they have also become pathogenic toward the genus *Homo* and, more recently, *Homo sapiens sapiens* (O'Brien et al., 2004; Tkachenko et al., 2016).

These pathogenic mycobacteria induce tuberculosis and leprosy, and they are classified as emergent prokaryotic agents of severe infectopathologies. They can maintain an active epidemic process in susceptible macroorganisms. In addition to epidemic variants of pathogenic mycobacteria, numerous nontuberculous mycobacteria with potentially pathogenic capabilities circulate among humans and animals. These bacteria cause sporadic cases of nontuberculous infections with low epidemic potential, also known as 'diseases of the future'. These infectopathologies are called mycobacterioses, and their agents belong to the category of potentially or conditionally pathogenic mycobacteria. These mycobacteria are also referred to as atypical, unclassified, or anonymous. The term 'atypical mycobacteria' is

the most widely used (Kassich et al., 2019; O'Brien et al., 2004).

Atypical mycobacteria belong to the phylum Actinobacteria, class Actinobacteria, order Corynebacteriales, family Mycobacteriaceae, genus *Mycobacterium*, and are characterized by significant species biodiversity.

According to Runyon's classification, atypical mycobacteria are divided into four groups. The most widespread in the environment of farm animals — and therefore the most frequent contaminants of livestock products — are group IV rapidly growing saprophytes, including *M. phlei*, *M. smegmatis*, *M. fortuitum*, *M. flavescens*, *M. vaccae*, *M. chelonae*, *M. peregrinum*, *M. diernhoferi*, and others (Hotsulia et al., 2021; Bihdan et al., 2018; Gomez-Buendia et al., 2024; Magee and Ward, 2015).

On artificial nutrient media, including MPA and MPB, atypical mycobacteria form macroscopically visible colonies within 7–10 days after inoculation from biomaterial, and in subcultures within 3–5 days at a temperature of 37–38°C in stationary aerobic conditions. Their colony biomass is abundant and forms in S- or R-types of colonial growth. Both pigmented and nonpigmented variants exist. Atypical mycobacteria are not demanding in cultivation conditions; for example, *M. phlei* can grow even at a temperature of 42°C. Colonies of some atypical species continue to grow at room temperature (20–22°C), though more slowly (Reber et al., 2016; Runyon, 1965; Tkachenko et al., 2010).

When animals are exposed to water or feed contaminated with atypical mycobacteria, they can develop short-term sensitization in the form of delayed-type hypersensitivity to tuberculin protein antigens. Bioassays on guinea pigs and farm animals repeatedly confirmed that oral or parenteral infection with atypical mycobacteria cultures results in typical sensitization to tuberculin sensitins. This is due to the antigenic similarity between tuberculous and nontuberculous atypical mycobacteria within the genus *Mycobacterium* (Abdulla et al., 2024; Atlas, 2010; Chen et al., 2025; Van Ingen et al., 2013; Solaghani et al., 2023).

Aim of the work was bacteriological study of milk and manure from clinically healthy dairy cows to detect genus *Mycobacterium* spp. and to characterize the basic properties of atypical mycobacteria circulating in the cows' environment, milk, and manure.

Materials and methods. Microbiological studies were conducted at the educational and research laboratory and vivarium of the Department of Infectious Diseases of the Faculty of Veterinary Medicine in Dnipro State Agrarian and Economic University.

Pre-seeding processing of cow feces was carried out according to the method of Alikaeva, using a 20% sulfuric acid solution with an exposure of at least 20 min, followed by thorough washing of the biomaterial with sterile saline. Milk samples were prepared following the official instructions for laboratory diagnosis of tuberculosis.

For the isolated mycobacterial cultures, species identification recorded the timing of initial growth, features of colony development, and morphology, pigmentation (or lack thereof). As differential diagnostic criteria, growth potential and accumulation of bacterial mass at a temperature of 25°C, 37°C, and 45°C were assessed, as well as haloresistance to 5% sodium chloride, growth in the presence of sodium salicylate, ability to reduce potassium tellurite, hydrolysis of Tween-80, catalase activity, and amidase activity.

Smear preparations from isolated mycobacterial cultures were stained by Ziehl–Neelsen and examined under immersion in a light microscope.

Simultaneous allergic testing of laboratory animals and dairy cows with PPD-tuberculin and atypical mycobacterial allergen (AAM) was carried out to determine the possibility of infection with pathogenic and nonpathogenic mycobacteria — that is, latent mixed mycobacteriosis — as well as the sensitizing potentials of field mycobacterial variants. Preparations were injected intradermally at a dose of 0.1 cm^3, with the injection site clipped and disinfected with 70% ethanol beforehand. Reactions were assessed after 24 h in guinea pigs, and after 48 h and 72 h in cows, by palpation and measuring skinfold thickness. In cows, an increase of ≥ 3 mm was considered positive; in guinea pigs, local signs of inflammation, edema, and necrosis were recorded.

Biological experiments were conducted on randomized short-haired guinea pigs weighing 300–400 g. They were infected with isolated field cultures of atypical mycobacteria to determine pathogenic potential and sensitizing properties. Two-week-old cultures suspended in saline were injected intradermally (inguinal region) at a dose of 1.0 mg. Clinical observation lasted for about two months, with simultaneous allergic tests using PPD-tuberculin and AAM performed 30 days and 15 days according to the instructions.

Results and discussion. At the beginning of the epizootic survey of farm animal health, simultaneous intradermal allergic testing of dairy cows was conducted using PPD-tuberculin for mammals and AAM produced by the Sumy Biofactory. After 72 h, examination of the reactions showed that all cows responded to both PPD-tuberculin and AAM. However, the intensity of skin reactions was significantly different: reactions to AAM were reliably stronger than those to PPD-tuberculin. The mean skinfold thickness after PPD-tuberculin injection was 6.3 ± 0.9 mm, while after AAM injection it was 22.6 ± 1.6 mm ($p \leq 0.05$).

During microbiological studies of general manure and milk samples from dairy cows, mixed cultures of atypical (nontuberculous) mycobacteria were isolated, including ten monocultures of a single species. The atypical mycobacteria were Gram-positive, acid-fast, polymorphic rods with pronounced saprophytic activity. They were not classified as pathogenic for humans or animals.

Following bacteriological identification, three cultures from the isolated pool of atypical mycobacteria were classified as *M. phlei*. When inoculated on Lowenstein–Jensen medium, primary growth of *M. phlei* was observed

as early as 3–5 days of incubation at a temperature of 37°C. *M. phlei* subcultures also produced colonial growth at a temperature of 25°C and 45°C, which is an important species marker. The colonies were dry, rough, wrinkled, and ranged in color from yellowish to orange or cream. Moderate, diffuse growth was observed on liquid media. Ziehl–Neelsen staining preserved the acid-fast properties, resulting in the characteristic ruby-red color of mycobacteria, along with a non-granular cytoplasm. Gram staining revealed large, weakly stained, light-violet, polymorphic rods with rounded edges, sometimes slightly curved, and non-granular cytoplasm (Fig. 1).

Biochemical analysis revealed catalase activity and the ability to reduce nitrates to nitrites, with the formation of niacin and nitrate reductase. Culture growth was observed on glycerol agar and simple nutrient media without the addition of inhibitors. The cultures demonstrated resistance to the main anti-tuberculosis drugs (isoniazid, ethambutol, rifampin), which is typical of non-pathogenic and rapidly growing atypical mycobacteria of Runyon group IV, and particularly *M. phlei.*

To assess pathogenic potency, a bioassay was conducted on guinea pigs. The animals were infected intradermally with a suspension of mixed *M. phlei* cultures and observed for 45 days. During this period, no clinical signs of the disease, such as weight loss, fever, or behavioral changes, were observed. After one month, some of the animals were sacrificed, and postmortem examination was performed. Autopsy revealed no pathological changes in the internal organs characteristic of tuberculosis infection.

An intradermal allergic test with PPD-tuberculin for mammals on 30th day after infection showed no reaction of the macroorganism to the tuberculous sensitin, which confirms the absence of sensitization of guinea pigs to the antigens of *M. phlei* and their inability to induce an immune response typical of tuberculous mycobacteria.

To assess the immunobiological properties of *M. phlei* in guinea pigs, 45 days after intradermal infection with the culture, simultaneous allergic diagnostics were performed using standard PPD-tuberculin for mammals and allergens from atypical mycobacteria. The following indicators were recorded: upon administration of PPD-tuberculin to mammals, there was no reaction; the papule diameter was between 0.1 mm and 0.9 mm; and there was no hyperemia or necrosis. This can be interpreted as an absence of sensitization to the antigenic complexes of pathogenic mycobacteria. Upon administration of AAM, some animals exhibited a mild local reaction with papules measuring up to 3 mm in diameter. There were no signs of systemic inflammation or necrosis.

This indicates partial immunological rearrangement induced by *M. phlei*, but its antigenic structure differs significantly from that of pathogenic mycobacteria. The simultaneous allergy test showed that infection with *M. phlei* does not lead to cross-sensitization to tuberculous sensitin and does not produce false-positive results in allergic intradermal diagnostics of tuberculosis, which is of great diagnostic importance for both veterinary and human medicine when interpreting allergy test results for tuberculosis and mycobacterioses.

Thus, the biological properties of isolated *M. phlei* cultures confirm their saprophytic nature. Field variants of *M. phlei* pose no epidemiological danger but must be differentiated in laboratory diagnostics of tuberculosis and mycobacterioses.

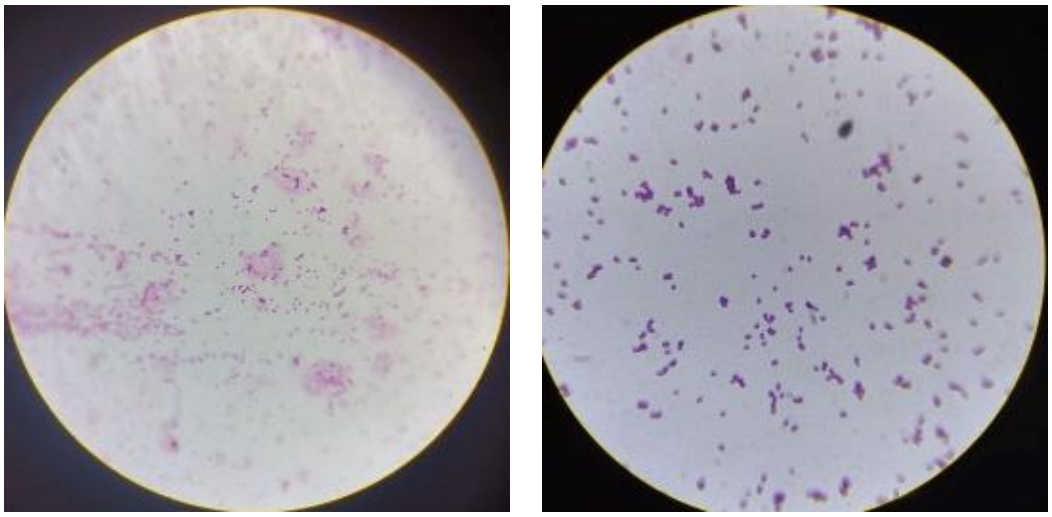
From manure, three pure cultures of *M. vaccae* and four cultures of *M. terrae* were isolated. They had very similar morphotinctorial properties and were obtained from the same manure samples. *M. vaccae* and *M. terrae* are known to be ubiquitous environmental saprophytes with pronounced immunomodulatory potential regarding the nonspecific resistance of macroorganisms. These bacteria are part of the dynamic pool of aerated soil microbiomes and are almost always found on the bodies of clinically healthy cows and in their manure. Thus, they serve as bioindicators of environmental biosafety.

Cultures of *M. vaccae* grew on Löwenstein–Jensen medium at a temperature of 25°C and 37 °C, but not at a temperature of 45°C or 4–6°C. Primary growth appeared on days 8–11 as dry, rough, wrinkled colonies with a yellow or orange tint. In liquid media, moderate diffuse turbidity was noted. With Ziehl–Neelsen staining, acid-fastness was confirmed: ruby-red coloration typical of mycobacteria, but with non-granular cytoplasm. With gram staining, *M. vaccae* appeared as weakly stained light-violet polymorphic large rods with rounded edges, sometimes slightly curved, with non-granular cytoplasm.

Biochemical tests revealed catalase activity, the capacity to reduce nitrates to nitrites, and the synthesis of niacin and nitrate reductase. *M. vaccae* grew well on glycerol agar and simple nutrient media at a temperature of 37°C. It tolerated 5% NaCl and grew in media with sodium salicylate. *M. vaccae* also hydrolyzed Tween-80.

Cultures of *M. terrae* exhibited morphotinctorial, cultural, and biochemical properties similar to those of *M. vaccae*. However, their colonies on Löwenstein–Jensen medium were nonpigmented and gray-white (Fig. 2). They were not resistant to 5% NaCl and did not exhibit amidase activity.

The biological properties of *M. vaccae* and *M. terrae* were studied in a traditional bioassay on guinea pigs. After infection with a standard infective dose, the animals were observed for 45 days. No pathological phenomena were recorded; the guinea pigs actively ate, were mobile, and well-nourished. After a month, simultaneous allergic testing was performed with standard PPD-tuberculin for mammals and AAM. The results were as follows: upon PPD-tuberculin administration, the reaction was practically absent. In some animals, slight edema at the injection site and small papules of 0.1–0.6 mm without hyperemia or necrosis were noted. This can be interpreted as an absence of sensitization to the antigenic complexes of pathogenic mycobacteria. With AAM administration, some animals showed a weak local reaction with papules up to 3 mm without systemic inflammation or necrosis.



a b

Figure 1. Microscopy of *M. phlei* culture, Ziehl–Neelsen (a) and Gram (b) staining.

Figure 2. Culture of *M. terrae* on selective nutrient medium.

As with *M. phlei*, the results suggest partial immunological rearrangement caused by antigenic commitment induced by atypical mycobacteria. However, the antigenic structures of *M. vaccae* and *M. terrae* differ significantly from those of pathogenic mycobacteria, preventing cross-sensitization with antigens of both pathogenic and nonpathogenic atypical mycobacteria.

A microbiological examination of milk and cow manure, using standard methods to isolate and identify mycobacterial prokaryotes, revealed their ubiquity, nonpathogenicity, and relative immunological activity when contaminating the internal environment of a macroorganism. It should be noted, however, that their presence in animals is temporary. Saprophytes cannot survive long in the internal environment and are transient components of the normal microbiota of the animal body.

Conclusions. 1. Milk from clinically healthy, tuberculin-negative cows contained no pathogenic mycobacteria, but was contaminated with various nonpathogenic atypical species.

2. Manure from healthy cows was contaminated with saprophytic atypical mycobacteria; no pathogenic strains were isolated.

3. Ubiquitous atypical nonpathogenic mycobacteria are natural co-inhabitants of livestock environments and products. They do not cause disease but can induce transient false allergic reactions to mycobacterial sensitins.

4. These saprophytes are nonpathogenic for mammals, but through transient contact with the immune system, they may cause false-positive skin reactions without pathological changes.

Prospects for further research. Atypical mycobacteria are ubiquitous environmental and animal-associated microbes that are widespread contaminants of livestock and their products. Their phylogenetic similarity to pathogenic species presents challenges in diagnosis. Studying the biological properties of these environmental mycobacteria is essential to improving the laboratory detection and identification of mycobacteria.

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STUDY OF THE BACTERICIDAL ACTIVITY OF THE 'DEZV ULTRA' DISINFECTANT AGAINST MYCOBACTERIA

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Summary. Timely and effective disinfection of livestock facilities and the environment is essential for maintaining bovine tuberculosis control. Currently, a significant number of disinfectants are used for disinfection, but not all of them have a bactericidal effect against tuberculosis pathogens and atypical mycobacteria at concentrations and exposures recommended by developers. The introduction of new disinfectants into veterinary practice is not possible without first determining their biocidal properties against zoonotic pathogens, including pathogenic and atypical mycobacteria, in the laboratory. The bactericidal activity of the disinfectant 'DezV Ultra' was determined using the suspension method and on test objects using test cultures of mycobacteria (*M. bovis, M. avium, M. fortuitum*). The quality of disinfection was tested on laboratory animals. The study was conducted at the Laboratory of Tuberculosis of the National Scientific Center 'Institute of Experimental and Clinical Veterinary Medicine'. According to the results of the studies, the disinfectant 'DezV Ultra' was found to destroy the tuberculosis pathogens *M. bovis* and *M. avium* on test objects with a biological load at concentrations of 2.0% and 3.0% with exposures of 24 h and 3 h, respectively. The bactericidal effect of 'DezV Ultra' against *M. fortuitum* in a suspension application was observed at a concentration of 1.0% for 24 h, 2.0% for 5 h, and 3.0% for 3 h. Conclusions: The disinfectant 'DezV Ultra' is recommended for the preventive and mandatory disinfection of premises in agricultural enterprises, regardless of whether they are considered safe or high-risk for bovine or poultry tuberculosis

Keywords: *Mycobacterium*, disinfection, glutaraldehyde, test objects, guinea pigs

Introduction. Tuberculosis is an infectious disease that poses a particular danger to farm animals, wild animals, and humans. It is characterized by the formation of avascular nodules (tubercles) in various organs with caseous necrosis, and it usually progresses chronically.

Producing high-quality livestock products poses many challenges to veterinary medicine. One important challenge is developing modern methods to diagnose and prevent particularly dangerous diseases, such as tuberculosis (TB).

According to a 2024 report by the World Health Organization, ending the global tuberculosis epidemic remains a distant goal. The number of new tuberculosis cases continues to slowly grow worldwide. In 2020, 10.1 million people contracted tuberculosis; in 2021 — 10.4 million; and in 2023 — 10.8 million. In 2023, global TB deaths were estimated at 1.25 million, 1.09 million of which were among people without HIV and 161,000 of which were among people with HIV (WHO, 2024).

In recent years, Ukraine has experienced a severe tuberculosis epidemic. According to the WHO, the estimated incidence of TB (new cases per 100,000 people) increased by more than 5% in Ukraine between 2015 and 2023. In 2024, the Center for Public Health of the Ministry of Health of Ukraine reported that the number of newly registered TB cases, including relapses, was 18,140, or 44.2 per 100,000 people. Incidence rates were recorded in all 24 regions of Ukraine, ranging from 32.0 to 86.2 per 100,000 people. The highest number of patients was recorded in Poltava Region (61.7 people per 100,000), Zakarpattia Region (69.6 people per 100,000), Kirovohrad Region (75.4 people per 100,000), Odesa Region (79.7 people per 100,000), and Dnipro Region (86.2 people per 100,000) (WHO, 2024; PHCMOHU, 2025).

Regarding the epizootic situation of bovine tuberculosis (TB), more than 50 million cattle worldwide are infected, resulting in about $3 billion in economic losses per year. In some parts of Africa, the infection rate can be as high as 50%. The disease is endemic in Central and South America, where it is particularly prevalent in dairy cattle. Thanks to preventive and rehabilitative measures implemented in most European Union countries, cattle herds have recovered from this infection. However, sporadic cases of the disease in healthy herds and recurrence of the infection in previously recovered herds are still reported. Sporadic outbreaks of bovine tuberculosis in Europe, Canada, and the United States typically occur in areas where livestock come into contact with wildlife populations (WOAH, 2025; Milián-Suazo et al., 2022).

European countries use a full range of microbiological and serological tests, and also molecular genetic methods (PCR) to monitor animal welfare and conduct monitoring studies. These methods allow for the timely identification of pathogens and the source of infection (WOAH, 2020, 2023, 2024).

According to official data, Ukraine is free of bovine tuberculosis. However, the migration of people, wild animals, and domestic animals transported from occupied territories to other regions without prior examination or quarantine restrictions may complicate the epidemic and epizootic situation. Additionally, the failure to fully implement veterinary and sanitary measures may contribute to this complication.

One of the main factors in the transmission of tuberculosis pathogens is the environment, which allows them to remain viable and retain virulence. To break the transmission mechanism, all potentially contaminated environmental objects must undergo high-quality cleaning and disinfection.

The timely and effective disinfection of livestock premises and the environment is crucial to the system of measures for preventing and controlling tuberculosis in farm animals. Inadequate disinfection can cause new and recurring tuberculosis outbreaks.

The primary objective of disinfection is to destroy pathogens in the animal environment and prevent the occurrence and spread of the disease (Paliy et al., 2020; Zavgorodniy et al., 2013).

In animal husbandry, disinfectants containing phenols, chlorine, alkalis, aldehydes, and acids are used (Paliy, Stegniy and Vedmid, 2016).

The Ukrainian market currently offers a wide range of disinfectants. However, most of these disinfectants are intended for human medicine and are not effective when used by veterinary specialists. This is due to significant bacterial contamination of veterinary objects, a high biological load, and other factors. Using such preparations does not lead to the destruction of microorganisms. Bacteriostatic concentrations of disinfectants only slightly decrease their number for a short period (Paliy et al., 2020; Zavgorodniy et al., 2013; Paliy, Stegniy and Vedmid, 2016). Consequently, various countries around the world are searching for and developing new disinfectants with a broad spectrum of bactericidal action that comply with environmental safety requirements and are not excessively toxic.

The study **aimed** to examine the bactericidal activity of the disinfectant 'DezV Ultra' against *Mycobacterium fortuitum*, *M. bovis*, and *M. avium.*

Materials and methods. The study used the disinfectant 'DezV Ultra', which contains the active ingredient glutaraldehyde. The bactericidal activity of 'DezV Ultra' was tested on the tuberculosis pathogens *M. bovis* (strain Vallee), *M. avium* (strain IECVM UAAS), and atypical mycobacteria (*M. fortuitum* strain 122). Mycobacterial cultures were used that were stored in the museum collection of the Laboratory of Tuberculosis of the National Scientific Center 'Institute of Experimental and Clinical Veterinary Medicine' (Kharkiv, Ukraine). The mycobacterial cultures were grown on Pavlovsky potato medium for 30 days, 20 days, and 10 days, respectively, at a temperature of 37.5 ± 0.5°C. Experiments used bacterial mass from non-inactivated test cultures of mycobacteria with typical tinctorial, cultural, and biological properties. The bactericidal effect of 'DezV Ultra' on the tuberculosis pathogens *M. bovis, M. avium,* and *M. fortuitum* was determined by three methods: suspension, contact-suspension *in vitro*, and biological (*in vivo*) (Zavgorodniy et al., 2007).

In vitro studies. The initial bactericidal activity of the drug was determined using the suspension method with a fast-growing culture of atypical mycobacteria of the species *M. fortuitum.*

Working solutions of the disinfectant 'DezV Ultra' were prepared at concentrations of 0.5%, 1.0%, 2.0%, and 3.0%. 10 cm^3 of each solution was added to 20.0 cm^3 vials. Then, 0.2 cm^3 of *M. fortuitum* suspension was added to each bottle with the disinfectant using a sterile pipette. The suspension was added in concentrations of 2×10^9, 1×10^9, and 1×10^6 bacterial cells in 1.0 cm^3 of sterile 0.85% sodium chloride solution. The vial contents were thoroughly mixed and kept at the specified exposure times (1 h, 3 h, 5 h, and 24 h). After exposure, 10.0 cm^3 samples were transferred from the vials to centrifuge tubes and centrifuged at 3,000 rpm for 15 min. To control for the bactericidal action of the test preparation, a suspension of the test culture was used in which 10.0 cm^3 of sterile isotonic sodium chloride solution was added instead of the disinfectant solution. For the negative control, the test culture was treated with a 3.0% formaldehyde solution at the same concentrations.

To stop the action of the disinfectant in the test vials, the sediment formed after centrifugation, as well as the control samples, were washed twice with a sterile isotonic solution by repeated centrifugation. The sediment was resuspended by adding 5.0 cm^3 of a 0.85% sodium chloride solution. After that, the resulting sediment suspension was inoculated onto a nutrient medium for the cultivation of mycobacteria (n = 5). The tubes with inoculations were cultivated in a thermostat at a temperature of 37.5 ± 0.5°C for 90 days, and the growth of cultures was recorded every 5–7 days.

Based on the results obtained for *M. fortuitum*, the parameters (concentration and exposure) of the disinfectant's disinfecting action were further determined by the contact-suspension method. This method used *M. bovis* and *M. avium* at a concentration of 2×10^9 bacterial cells in 1.0 cm^3 of 0.85% sodium chloride solution on test objects (batiste, wood, and ceramics) with a biological load (manure). A mixture containing 1.0 cm^3 of a suspension of test cultures of the tuberculosis pathogens *M. bovis* and *M. avium* and 0.5 cm^3 of sterile manure was applied to each test object for this purpose. After 12 h, the test objects were treated with working solutions of the disinfectant. For the positive control, the test objects were treated with a sterile isotonic sodium chloride solution instead of the disinfectant. For the negative control, the test objects were treated with a 3% formaldehyde solution.

After the specified exposure period, scrapings were taken from each of the experimental and control test objects. The scrapings were washed with a sterile isotonic solution in Petri dishes. The contents of the dishes were then transferred to centrifuge tubes and centrifuged at 3,000 rpm for 15 min. To neutralize the drug's effect, the sediment was washed twice with sterile isotonic solution by centrifugation. The sediment from the experimental and control samples was then resuspended in 5.0 cm^3 of sterile isotonic solution and inoculated onto a dense nutrient medium to cultivate mycobacteria.

The test tubes containing the cultures were stored in a thermostat at a temperature of 37.5 ± 0.5°C for 90 days. Growth was recorded every five to seven days.

In vivo studies. The biological study was conducted on six clinically healthy guinea pigs (three in the experimental group and three in the control group), as well as two experimental rabbits and two control rabbits. None of the animals reacted to the tuberculin (PPD) test before the experiment. The laboratory animals were injected with a decontaminated suspension of sediment obtained after treating the experimental and control test objects with 2.0% 'DezV Ultra' for 24 h. The guinea pigs were inoculated subcutaneously in the groin with a 1.0 cm^3 dose of the decontaminated *M. bovis* test culture suspension. The rabbits were administered a 1.0 cm^3 dose of the *M. avium* test culture suspension intravenously.

The laboratory animals were observed for 90 days. During this time, the animals underwent an intradermal tuberculin test once every 30 days (three times total). Animals that died during the experiment or were euthanized after its completion were examined for tuberculosis using pathological and cultural methods.

Statistical analysis was performed by counting colony-forming units (CFUs). The results were processed by methods of variation statistics. To compare mean values Student's *t*-test was used (Van Emden, 2019).

Results. The results of a preliminary study of the bactericidal activity of 'DezV Ultra' against atypical mycobacteria (*M. fortuitum*) using the suspension method are shown in Table 1.

Analysis of the results shows that the drug 'DezV Ultra' exhibited a bacteriostatic effect against *M. fortuitum* at a concentration of 1×10^6 cells when a 0.5% solution was used for 3–24 h, a 1.0% solution for 1–3 h, and a 2.0% solution for 1 h. Regarding *M. fortuitum* with initial concentrations of 1×10^9 and 2×10^9 cells, 'DezV Ultra' had only bacteriostatic properties when used at a concentration of 0.5% for exposure times of 3–24 h, 1.0% for 1–5 h, 2.0% for 1–3 h, and 3.0% for 1 h.

Table 1 — Results of cultural studies of the bactericidal activity of 'DezV Ultra' against *M. fortuitum*, M ± m, n = 5

Mode of application		Concentration, CFU/cm^3				
		Experiment			Control	
Concentration of disinfectant solution, %	Exposure, h	1×10^6	1×10^9	2×10^9	Positive control	Negative control
0.5	1	90.2 ± 3.6	102.8 ± 5.2	118.6 ± 4.8	92.3 ± 2.8/ 1×10^6 106.8 ± 1.8/ 1×10^9 119.6 ± 2.2/ 2×10^9	–
	3	56.8 ± 4.8*	96.8 ± 5.5*	100.4 ± 6.3*		–
	5	26.8 ± 2.4*	72.2 ± 4.8*	88.2 ± 5.6*		–
	24	19.8 ± 6.0*	46.0 ± 3.2*	48.0 ± 4.4*		–
1.0	1	12.6 ± 2.4*	52.6 ± 4.8*	58.8 ± 4.2*		–
	3	8.5 ± 1.4*	36.2 ± 2.2*	46.2 ± 3.6*		–
	5	–	8.4 ± 1.4*	9.6 ± 1.6*		–
	24	–	–	–		–
2.0	1	6.5 ± 0.5*	28.8 ± 1.8*	38.4 ± 2.4*		–
	3	–	15.6 ± 1.9*	17.6 ± 0.9*		–
	5	–	–	–		–
	24	–	–	–		–
3.0	1	–	4.0 ± 0.7	4.8 ± 1.0		–
	3	–	–	–		–
	5	–	–	–		–
	24	–	–	–		–

Notes: * — $p < 0.05$ relative to control; '–' — no growth.

The bacteriostatic effect of the drug was manifested by a decrease in the number of colonies and their diameter, a delay in the appearance of primary colonies by 1–2 days, and at a 3% concentration of the drug by up to 4 days, compared with the positive control. It should be noted that there was almost no difference in the rate and intensity of colony growth after exposure to a 0.5% solution of 'DezV Ultra' for 1 h and the growth of *M. fortuitum* in control tubes that were not exposed to the drug. The results of the experiment show that the number of colonies that grew after exposure to the disinfectant depended on the initial concentration of mycobacteria. Under conditions of equal concentration and exposure to the preparation, a greater number of mycobacteria remained viable at a higher initial concentration of bacterial cells, which was reflected in the number of colonies that grew. Thus, when using an initial concentration of test cultures of 1×10^6 cells, compared to an initial concentration of 2×10^9 cells, the difference in colonies that grew after the drug was applied ranged from 1.3 (0.5% — 1 h) to 5.4 (1% — 3 h) times, and as the concentration and exposure of the drug increased, the difference in the number of colonies also increased.

When used as a suspension, 'DezV Ultra' exhibited bactericidal properties against *M. fortuitum* at an initial concentration of 1 million cells at concentrations of 1% for exposures of 5–24 h, 2% for exposures of 3–24 h, and 3% for exposures of 1–24 h. Regarding concentrations of *M. fortuitum* at 1 and 2 billion cells, the drug exhibited bactericidal activity at 1.0% for a 24-hour exposure; 2.0% for a 5- or 24-hour exposure; and 3.0% for a 3-, 5-, or 24-hour exposure.

Following positive preliminary study results, the final determination of the disinfecting action of the 'DezV Ultra' product was conducted on tuberculosis pathogens (*M. bovis* and *M. avium*) using test objects, including wood, ceramic tiles, and batiste.

Manure was used as a biological load for this purpose. Due to the risk of high concentrations of mycobacteria or tuberculosis pathogens accumulating in farm buildings and on premises, the maximum concentrations of *M. bovis* and *M. avium* (2×10^9 in 1.0 cm^3) were used to study the product's bactericidal activity on the test objects. The results of these studies are presented in Table 2.

Table 2 — Results of determining the bactericidal effect of the 'DezV Ultra' drug on *M. bovis* and *M. avium* cultures on test objects

Mode of application		Test-object			Control	
Concentration of disinfectant solution, %	**Exposure, h**	**wood**	**batiste**	**ceramic tiles**	**Positive control**	**Negative control**
M. bovis						
1	5	9.8 ± 1.2*	8.5 ± 0.9*	–	78.9 ± 4.5/wood 92.5 ± 6.2/batiste 86.8 ± 7.4/ceramics	–
	24	7.6 ± 2.4*	–	–		–
2	5	6.8 ± 0.8*	–	–		–
	24	–	–	–		–
3	3	–	–	–		–
	5	–	–	–		–
	24	–	–	–		–
M. avium						
1	5	9.4 ± 2.4*	8.8 ± 2.6*	–	88.2 ± 6.8/wood 104.5 ± 8.4/batiste 106.8 ± 8.6/ceramics	–
	24	8.0 ± 1.6*	–	–		–
2	5	7.6 ± 0.9*	–	–		–
	24	–	–	–		–
3	3	–	–	–		–
	5	–	–	–		–
	24	–	–	–		–

Notes: * — $p < 0.05$ relative to control; '–' — no growth.

The data in Table 2 show that the disinfectant 'DezV Ultra' at a concentration of 1.0% with an exposure time of 5–24 h and at a concentration of 2.0% with an exposure time of 5 h does not disinfect wood contaminated with *M. bovis* and *M. avium* pathogens. As for contaminated batiste, a 1% solution of the product also failed to inactivate the pathogens after 5 h of exposure. However, at all concentrations and exposure times, this product exhibited bactericidal activity on ceramic tiles contaminated with *M. bovis* and *M. avium*. This difference in the results of decontamination of wood and ceramics is due to the structure of the test objects, namely their porosity. The more pores there are in a test object, the more difficult it is to decontaminate it. When the preparation was used at a concentration of 2.0% for 24 h of exposure and at a concentration of 3.0% for 3–24 h of exposure, all test objects were decontaminated.

Biological testing confirmed the bactericidal properties of the experimental disinfectant at a concentration of 2.0% against *M. bovis* and *M. avium* pathogens after 24 h of exposure. To determine the quality of decontamination, guinea pigs and rabbits were injected with swabs from test objects. Only laboratory animals from the control groups reacted positively to intradermal administration of tuberculin (PPD) for mammals and birds. During the pathological examination of guinea pigs infected with wood swabs (positive control), lesions characteristic of tuberculosis were found, and in rabbits, the course of the infectious process was of the Yersin type. Cultural studies of pathological material taken from experimental and control animals isolated *M. bovis* and *M. avium* cultures only from animals in the control groups. The experimental animals did not respond to intradermal administration of tuberculins, and no mycobacterial cultures were isolated from the biomaterial after the experiment was completed.

Conclusions. 1. Bacteriological and biological studies of the bactericidal properties of 'DezV Ultra' concerning mycobacteria have established that this disinfectant destroys the tuberculosis pathogens *M. bovis* and *M. avium* at a concentration of 2.0% after 24 h of

exposure and at a concentration of 3.0% after 3–24 h of exposure.

2. 'DezV Ultra' can be used to preventively and obligatorily disinfect premises in agricultural enterprises with or without tuberculosis in cattle or poultry. It can be used in the form of a 2.0% aqueous solution for 24 h or a 3.0% solution for 3–24 h, at a rate of 1,000.0 cm^3 per 1.0 m^2.

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