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Exposure to Disinfectants of Various Chemical Nature on the Culture of Pathogenic Leptospira

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Abstract. Infectious diseases cause substantial economic damage to livestock farms, so there is a constant search for new means of preventing diseases, especially disinfectants. Analysis of the scientific literature indicates a substantial problem of leptospirosis in Ukraine and there are virtually no data on the use of complex oxidising drugs for its prevention. The purpose of the work was to examine the effect of Biolide (active substances hydrogen peroxide, lactic and supralactic acids) and Diolide disinfectants (active substances sodium chlorite and sodium chloride) on the causative agents of leptospirosis. The stability of eight pathogenic Leptospira cultures of different ages circulating in Ukraine and their growth properties were tested by adding different concentrations of these disinfectants to them. The results obtained were statistically analysed in the Epitools – Epidemiological Calculators software. Effective concentrations and exposures of Biolide and Diolide for use in preventive and forced disinfection in leptospirosis were determined. As a result of studies on the effect of both disinfectants on 7-, 10- and 15-days Leptospira test cultures, no differences were recorded between the indicators of their accumulation (number of microbial cells/cm^3). Therefore, the results obtained for cultures of different ages were considered as repeatability. It is proved that for preventive and forced disinfection in leptospirosis, a 0.55% solution of Biolide is recommended for use at an exposure of 30 minutes at a temperature of 24°C. If the exposure period is increased to 60 minutes, it is allowed to reduce the concentration of the product to 0.185%. Regarding the drug "Diolide", it is recommended to use it in this zoonosis in a dilution of 200 mg/l (concentration of 0.08% of the active substance) during exposure for 15 minutes at a temperature of 24°C. If the exposure period is increased to 30 minutes, it is allowed to reduce the dilution of the drug to 50 mg/dm^3 (concentration of 0.02% of the active substance). In addition, it was determined that both disinfectants completely inhibit the growth of pathogenic cultures of Leptospira. The practical value of the study is to prove the possibility of using complex disinfectants based on oxidising agents for the prevention of leptospirosis

Keywords: veterinary medicine, leptospirosis, disinfection, Biolide, Diolide, bactericidal

Introduction

In January 1915, Japanese researchers Inada and Ido announced the discovery of the causative agents of Weyl's disease, which later became known as leptospirosis [1]. Leptospirosis is an infectious disease with a global spread, which is one of the most common (registered on all continents except Antarctica) and substantial in socio-economic terms natural focal zoonoses and poses a substantial danger to human and animal health (affects about 150 species of mammals) [2; 3]. Thus, about 0.5 million cases of human leptospirosis per year are registered globally, and the mortality

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rate ranges from 5% to 15% [4; 5]. Animals with leptospirosis develop non-sterile immunity. In addition, the prevalence of the disease is facilitated by the variability of its etiological structure among different animal species, both within countries and regions, districts, etc. [6]. This fact is primarily explained by the extreme variability of pathogens (there are about 250 serovars of Leptospira) [2; 7].

All of the above contributes to the constant search for new effective measures for the prevention of leptospirosis, namely vaccines and disinfectants that would destroy the causative agent of the disease during its stay in the environment after isolation from the host (sick animal or human) [8].

For effective prevention, priority should be paid to the biology of the causative agent of the disease. Thus, according to recent studies, Leptospira are tightly coiled spirochetes, usually ranging in size from 0.1 by 6 microns to 0.1 by 20 microns, but a separate culture may contain longer cells [9]. Microorganisms are mobile, have rounded ends, which are usually bent in the form of hooks. Leptospira, like other spirochetes, have a typical double membrane structure, in which the cytoplasmic membrane and the peptidoglycan cell wall are closely connected and overlapped by an outer membrane. These microorganisms are Gram-negative obligate aerobes with an optimal growth temperature of 28 to 30°C [9; 10]. Given the hydrophilicity of the pathogen (the vital activity of Leptospira substantially depends on the availability of water sources), the number of outbreaks of the disease increases substantially after natural disasters (hurricanes, floods, etc.) [11]. This indicator also depends on the climate – the wetter it is, the more outbreaks there are [12].

It has been experimentally proven that pathogenic Leptospira cannot circulate and survive in nature without the involvement of host organisms, and the main transmission routes are contaminated environmental objects [13; 14]. For example, in soil and water, these microorganisms can survive for 16-28 days, after which molecular genetic diagnostic methods begin to give a negative result [15]. Therefore, to interrupt the epizootic chain of infection, Leptospira must be destroyed on contaminated surfaces, bedding, feeders, drinkers, etc.

Since Leptospira do not form spores and are relatively unstable in the environment, according to the recommendations of the World Organisation for Animal Health, the following chemicals/disinfectants affect them: 1.0% sodium hypochlorite, 70% ethanol, formaldehyde, detergents, quaternary ammonium compounds, iodine-based compounds, glutaraldehyde, and hydrogen peroxide [8]. Analysis of the scientific literature indicates that oxidising disinfectants, which include halogens, chlorine, iodine, bromine, and chlorine dioxide, and oxygen-releasing substances such as peracetic acid and hydrogen peroxide, are most common in animal and poultry farming [16]. The relevance of such drugs is promoted by their cost-effectiveness combined with the ease of their use, especially in the presence of animals. Most often, complex drugs based on lactic acid, quaternary ammonium compounds, hydrogen peroxide, and other non-toxic oxidising agents are used. These substances are cheap compared to other active substances, have a wide spectrum of bactericidal action and low corrosion activity [17]. Therewith, there are no complex drugs based on these compounds on the Ukrainian market for the disinfection of livestock premises from pathogens of leptospirosis.

However, effective use of the disinfectant in production conditions is possible with its through examination at the stage of laboratory and production tests. For the purpose of preventive and forced disinfection in the event of dangerous infectious diseases, a detailed analysis of the bactericidal effect of new findings in disinfectants, including in relation to leptospirosis, is necessary.

Considering all the above, *the purpose of the study* was to determine the effective concentrations and exposure of complex disinfectants "Biolide" and "Diolide" for use in preventive and forced disinfection in leptospirosis.

Materials and Methods

All studies, the results of which are presented in the study, were fully conducted based on the State Scientific and Research Institute of Laboratory Diagnostics and Veterinary and Sanitary Expertise (DNDILDVSE, Kyiv) during January-February 2022.

As a test culture for experiments, strains of eight serogroups of Leptospira were used, which most often cause leptospirosis among animals in Ukraine and are included in the diagnostic series of Leptospira for setting up a microagglutination reaction (PMA) on the territory of the state (Table 1).

Table 1. List of Leptospira strains and their serogroup and serovariant correspondence

No.	Serogroup	Serovar	Strain
1.	Sejroe	Polonica	493 Poland
2.	Hebdomadis	Kabura	Kabura
3.	Tarassovi	Tarassovi	Perepelicyni
4.	Pomona	Pomona	Pomona
5.	Grippotyphosa	Grippotyphosa	Moskva V
6.	Canicola	Canicola	Hond Utrecht IV
7.	Icterohaemorrhagiae	Copenhageni	M 20
8.	Australis	Bratislava	Yez bratislava

The experiment used 7- ("young" culture), 10- ("mature" culture), and 15-days ("old" culture) Leptospira test cultures with an accumulation of at least 80-100 million microbial cells/cm^3, typical morphology of mobile microorganisms.

The objects of research were Biolide disinfectants with hydrogen peroxide, lactic, and supralactic acids in the composition, and Diolide, the active ingredients of which are sodium chlorite and sodium chloride. The developer of both disinfectants is DNDILDVSE.

Preparation of working solutions of these disinfectants, and the selection of their concentrations and exposures necessary for research, was conducted in accordance with the leaflets-tabs to them. The method of sequential multiple dilutions was used to obtain the required concentrations [18].

Thus, for studies on the effect of Biolide disinfectant on Leptospira culture, 2.0 cm^3 of the environment of the Terskikh were introduced into each of the five glass test tubes 1.0 cm^3 of 10% disinfectant solution was added to the first test tube and thoroughly mixed. After that, 1.0 cm^3 was transferred from the first test tube content to the second, from the second to the third, etc. 1.0 cm^3 was removed from the fifth test tube. As a result, multiple dilutions were obtained 1 : 2, 1 : 4, 1 : 8, 1 : 16 and 1: 32 with a drug concentration of 3.33%, 1.11, 0.37, 0.123 and 0.041%. At the next stage, they were transferred to 5 sterile test tubes of 1.0 cm^3 each from contents from each dilution and added to them 1.0 cm^3 of culture of Leptospira of a certain age. Thus, working dilutions of the product in the mixture of 1.67%, 0.55, 0.185, 0.062, and 0.02% were obtained, respectively.

Regarding the study of the effect of Diolide on the stability of these crops, a uterine solution of a disinfectant with a chlorine dioxide content of 5000 mg/cm^3 was first prepared, for which 4 g of the drug was dissolved in 100.0 cm^3 of distilled water. An initial experimental concentration of 400 mg/cm^3 was then prepared, by adding 8.0 cm^3 of the uterine solution up to 92.0 cm^3 of distilled water. A series of multiple dilutions in 5 glass tubes was prepared as follows: 2.0 cm^3 was added to the first tube of solution with a working concentration of 400 mg/cm^3 and to the next 4 test tubes 1.0 cm^3 of the environment of the Terskykh was added. Then 1.0 cm^3 was transferred from the first test tube contained in the second, from the second to the third, etc. 1.0 cm^3 was removed from the fifth test tube. As a result, a series of multiple dilutions of 0.16%, 0.08, 0.04, 0.02, and 0.01% were obtained with a concentration of 400 mg/cm^3 of chlorine dioxide in them, 200, 100, 50, and 25 mg/cm^3. At the final stage, 1.0 cm^3 was added to each test tube with a culture of Leptospira of a certain age. After applying the culture, the working dilutions of the product in the mixture were 0.08%, 0.04, 0.02, 0.01, and 0.005% (200 mg /cm^3, 100, 50, 25, and 12.5 mg/cm^3 accordingly).

For the experiment on testing disinfectors for the growth of Leptospira culture, a Terskykh nutrient environment (pH 7.2-7.4) was prepared with the addition of 10.0% rabbit blood serum, which ensures optimal growth of these microorganisms. The accumulation of Leptospira was recorded visually in the dark field of view of the microscope at a magnification of 10x40 in accordance with international requirements [8].

All manipulations of the experiments were performed three times. The minimum bactericidal activity of the investigated agents was determined by the lowest concentration of drugs that inhibit the growth of Leptospira. The study was performed at exposures of 15, 30 and, if necessary, 60 minutes at room temperature.

Statistical processing of the obtained results in the course of the study was performed in the software Epitools – Epidemiological Calculators [19]. It was used to calculate confidence intervals (CI) for the obtained values at the probability level (P) of 0.95. Therewith, the Student's test was not calculated due to substantial deviations in numerical ranges (for example, the accumulation of Leptospira could be recorded in the range of 10-30 million microbial cells/cm^3).

Results and Discussion

As a result of studies on the effect of both disinfectants on 7-, 10-, and 15-days-old Leptospira test cultures, no difference was recorded between the indicators of their accumulation (number of microbial cells/cm^3). Therefore, the results obtained in the context of cultures of different ages were considered repeatability.

Systematised and visualised results of studies on the effect of various concentrations of Biolide on the resistance of Leptospira strains are presented in Table 2 and in Figure 1.

Table 2. Results of the effect of Biolide disinfectant on Leptospira culture at different concentrations and exposures (n = 9)

Exposure, min.	Biolide concentration, %	Leptospira serogroups (mil/mL)								Control (mil/mL)
		Sejroe	Hebdomadis	Tarassovi	Pomona	Grippotyphosa	Canicola	Icterohaemorrhagiae	Australis	
15	1.67	–	–	–	–	–	–	–	–	80-100
	0.55	–	5-10	–	0-5	–	–	0-5	10-20	80-100
	0.185	0-20	15-40	5-15	0-20	15-40	0-20	20-30	30-40	80-100
	0.062	5-40	50-60	15-20	30-50	30-50	30-40	40-60	50-60	80-100
	0.02	15-40	50-70	20-30	30-50	40-50	40-50	50-70	60-70	80-100

30	1.67	–	–	–	–	–	–	–	–	80-100
	0.55	–	–	–	–	–	–	–	–	80-100
	0.185	–	5-10	–	–	0-10	–	–	5-10	80-100
	0.062	0-30	40-50	15-20	20-40	20-40	20-30	40-50	50-60	80-100
	0.02	15-30	40-60	20	20-40	20-50	30-40	50-70	60-70	80-100
60	1.67	–	–	–	–	–	–	–	–	80-100
	0.55	–	–	–	–	–	–	–	–	80-100
	0.185	–	–	–	–	–	–	–	–	80-100
	0.062	0-20	30-40	0-10	10-40	10-30	0-30	20-50	30-60	80-100
	0.02	5-20	40-60	10-15	10-40	10-50	30	40-60	40-70	80-100

Note: in the table, the values of confidence intervals (Cl) relative to the number of Leptospira in the field of view of the microscope with a probability level of 0.95 are given, "–" – the absence of Leptospira in the field of view of the microscope

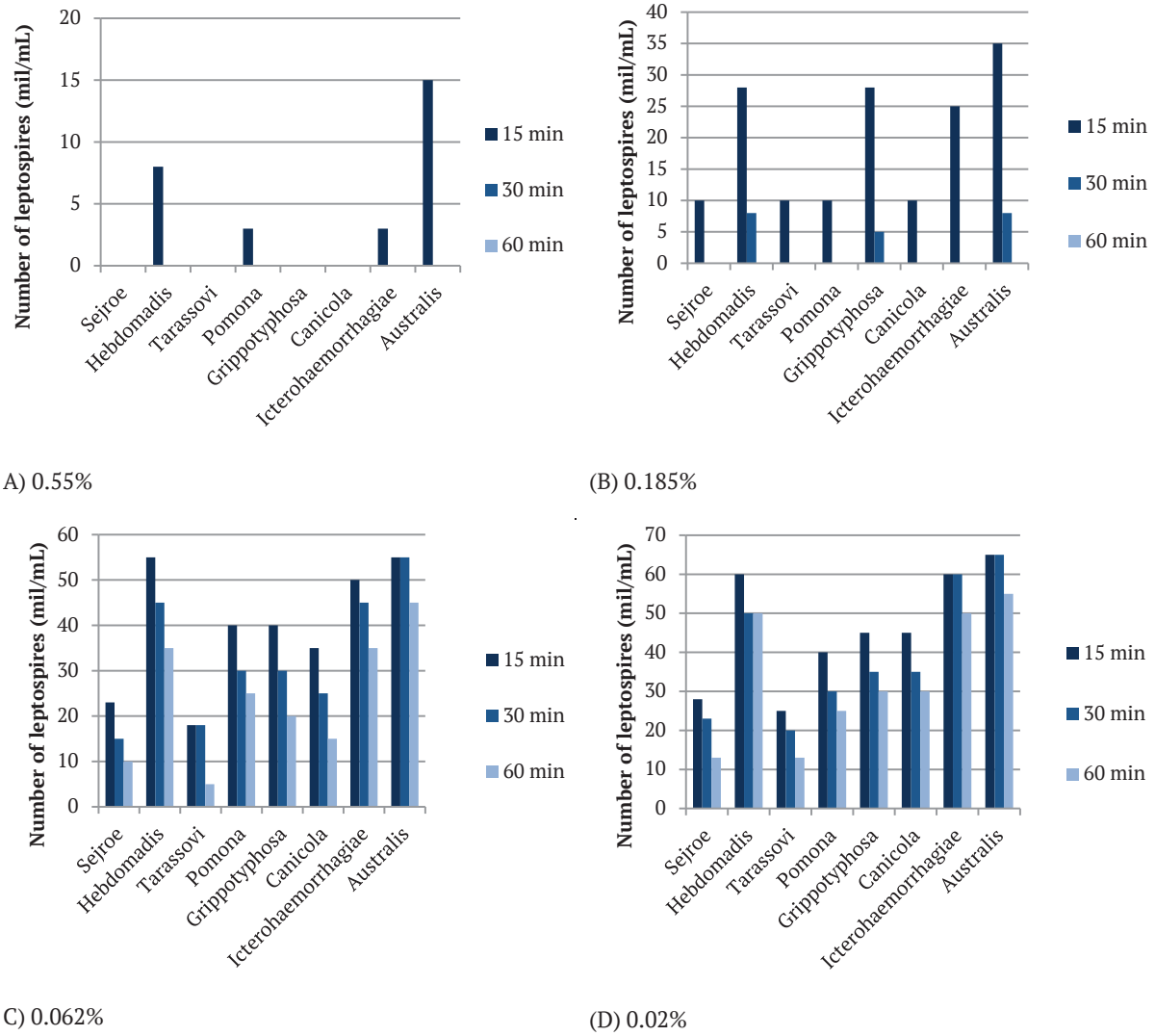


Figure 1. Dynamics of Leptospira registration due to exposure to different Biolide concentrations at exposures of 15-120 minutes

Analysing the obtained resistance indicators, it was determined that at drug concentrations of 1.67%, no microorganisms were registered in all the investigated cultures after 15 minutes of exposure. In the case of using a 0.55% solution, the accumulation of Leptospira in certain serogroups was 4-16 times less than in the control (native culture of Leptospira of 7-15 days of age without adding a disinfectant) or was not recorded at all. Thus, serogroups were the most sensitive to the action of a 0.55% solution of the drug "Biolide" under this exposure *Sejroe*, *Tarassovi*, *Grippotyphosa*, and *Canicola* (the micro-organisms were not visible at all in the field of view of the microscope.) In cultures *Pomona* and *Icterohaemorrhagiae* up to 5 million microbial cells/cm^{3}were registered. Therewith, the most persistent were *Hebdomadis* (5-10 million) and *Australis* (10-20 million microbial cells).

With a decrease in the concentration of the drug, the number of microbial cells in Leptospira cultures increased accordingly. Thus, when using a 0.185% solution – the average accumulation was 10-30 million microbial cells/cm,^{3}. In experimental cultures with 0.062 and 0.02% concentrations of the drug "Biolide" during 15-minute exposure, the accumulation of pathogenic cultures was quite substantial. Thus, in cases with serogroups *Icterohaemorrhagiae*, *Hebdomadis*, and *Australis* – this indicator was generally at the level of controls.

During exposure for 30 minutes, microorganisms were not recorded at all in all experimental samples with drug concentrations of 1.67 and 0.55%. In general, during this exposure, the amount of Leptospira in all cultures decreased slightly at different concentrations. In particular, the number of microorganisms substantially decreased in cultures when using the drug "Biolide" in the form of a 0.185% solution (Leptospira was detected only in samples with serogroups *Hebdomadis*, *Grippotyphosa*, and *Australis* (up to 10 mil/mL). Even in samples with a 0.02% solution, the accumulation rates were lower than in the controls.

Considering the data obtained, it was decided to continue the experiment with exposure for 60 minutes. As a result, pathogens were also not recorded in samples with a concentration of the drug "Biolide" of 0.185%. Therewith, with smaller dilutions of the drug, the accumulation indicators generally almost did not differ from those with previous exposure. Considering this, it was decided to stop the experiment.

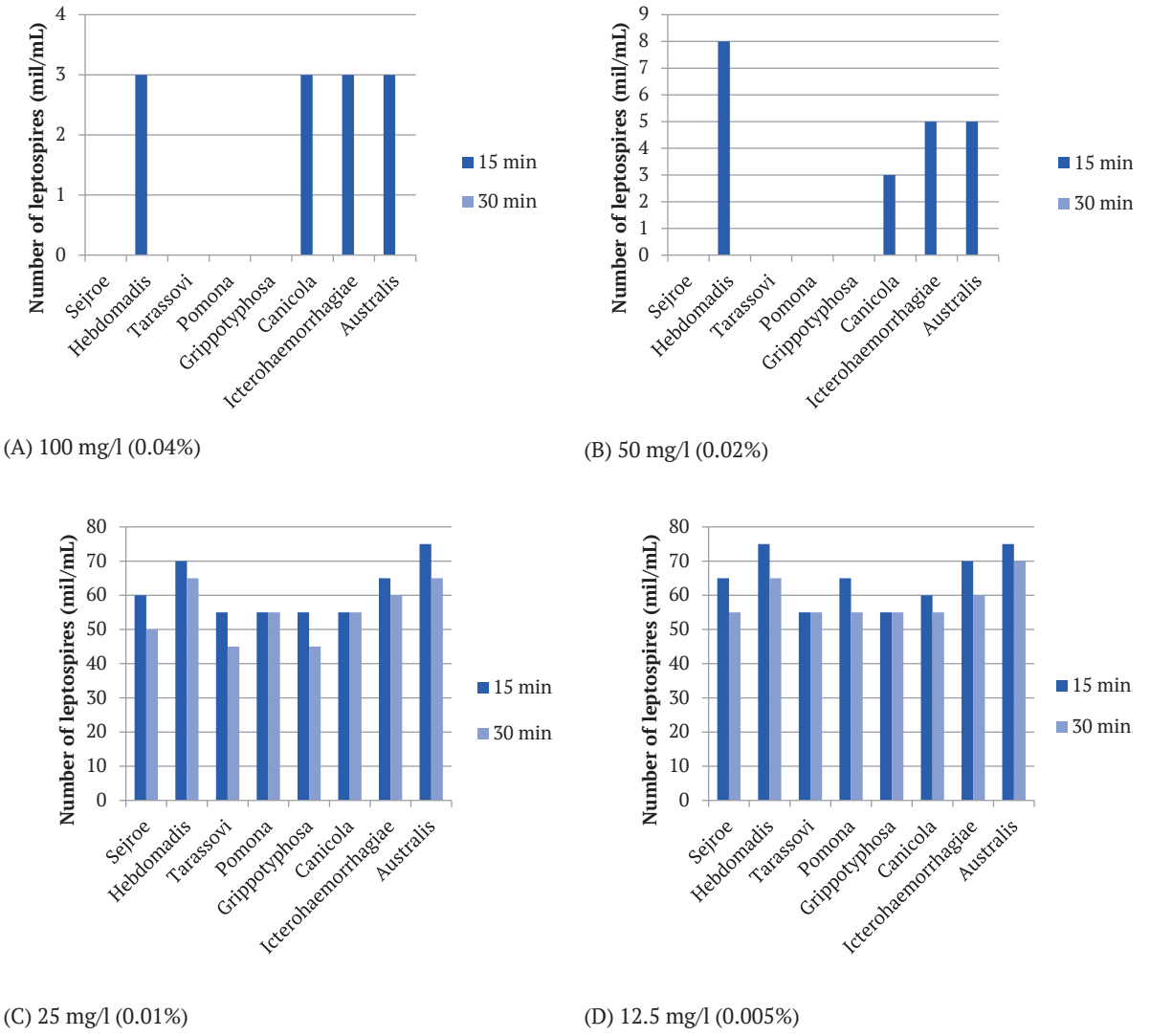
After systematising all the results obtained, it was found that serogroup cultures were the most resistant to low concentrations of the drug "Biolide" (0.02-0.062%). *Hebdomadis*, *Icterohaemorrhagiae*, and *Australis*.

The data obtained during the systematisation and visualisation of the results on the effect of various concentrations of Diolide on the resistance of Leptospira strains are presented in Table 3 and in Figure 2.

Table 3. Results of the effect of Diolide disinfectant on Leptospira culture at different concentrations and exposures (n = 9)

Exposure, min.	Dilution, mg/l (concentration, %)	Leptospira serogroups (mil/mL)								Control (mil/mL)
		Sejroe	Hebdomadis	Tarassovi	Pomona	Grippotyphosa	Canicola	Icterohaemo-rrhagiae	Australis	
15	200 (0.08)	–	–	–	–	–	–	–	–	80-100
	100 (0.04)	–	0-5	–	–	–	0-5	0-5	0-5	80-100
	50 (0.02)	–	5-10	–	–	–	0-5	0-10	10	80-100
	25 (0.01)	50-70	60-80	50-60	50-60	50-60	40-70	60-70	70-80	80-100
	12.5 (0.005)	60-70	60-90	50-60	60-70	50-60	50-70	60-80	70-80	80-100
30	200 (0.08)	–	–	–	–	–	–	–	–	80-100
	100 (0.04)	–	–	–	–	–	–	–	–	80-100
	50 (0.02)	–	–	–	–	–	–	–	–	80-100
	25 (0.01)	40-60	60-70	40-50	50-60	40-50	40-70	60	60-70	80-100
	12.5 (0.005)	40-70	60-70	50-60	50-60	50-60	40-70	60	60-80	80-100

Note: the table shows the values of confidence intervals (Cl) relative to the number of Leptospira in the field of view of the microscope with a probability level of 0.95, "–" – the absence of Leptospira in the field of view of the microscope



(A) 100 mg/l (0.04%)

(B) 50 mg/l (0.02%)

(C) 25 mg/l (0.01%)

(D) 12.5 mg/l (0.005%)

Figure 2. Dynamics of Leptospira registration due to exposure to different Diolide concentrations at 15 and 30 min exposures

Analysing the indicators of resistance of Leptospira culture to Diolide, its more pronounced inactivating effect was established than that of Biolide. This result indicates a substantial sensitivity of Leptospira to chlorine-containing compounds.

In particular, already at an exposure of 15 minutes when using the drug in a dilution of 200 mg/dm^3 (concentration of 0.08% for the active substance), no microorganisms were detected in any of the investigated cultures. In dilutions of Diolide 100 mg/dm^3 and 50 mg/dm^3 (0.04 and 0.02% active substance concentrations, respectively) single microorganisms were detected (5-10 mil/mL) in serogroup cultures *Hebdomadis*, *Canicola*, *Icterohaemorrhagiae*, and *Australis*. Therewith, when used during this exposure, smaller dilutions of the drug (12.5-25.0 mg/dm^3) indicators of microbial cell accumulation were within the limits of those in the controls.

After further exposure for 30 minutes, Leptospira was not detected at all in samples with Diolide dilutions of 50-200 mg/dm^3. As for the lower concentrations of the drug, as in the previous case, the accumulation of these microorganisms did not change substantially (on average, 50-60 mil/mL). Considering the result, it was decided not to continue the experiment.

Systematising the results obtained, it was found that the most resistant to low concentrations of Diolide (dilution 12.5-25.0 mg/dm^3, concentration 0.005-0.01%) identified serogroup cultures *Hebdomadis*, *Canicola*, and *Australis*.

In both experiments, the culture was incubated only at a temperature of 24°C, because Leptospira is sensitive to heat, which can inactivate them. Thus, according to the recommendations of the World Organisation for Animal Health, this type of microorganism is sensitive even to an increase in ambient temperature to a temperature of over 34°C [8]. Incubation was conducted at room temperature, since this allows the use of drugs in selected concentrations in livestock and poultry premises without the use of additional equipment.

Similar experiments with Leptospira culture have previously been conducted in Ukraine using disinfectants geocide (active ingredients are polyhexamethylene guanidine hydrochloride, benzalkonium chloride, and deltamethrin) and argicide (polyhexamethylene guanidine hydrochloride,

colloidal solutions of silver, and copper nanoparticles). Thus, it was experimentally established that the optimal concentration for the prevention of leptospirosis is a 0.55% solution of geocide with an exposure of 15 minutes. If the exposure period is increased to 30 minutes, it is allowed to reduce the concentration of the product to 0.02%. As for argicide, its optimal concentration for use is a 0.1% solution with an exposure of 60-75 minutes [20].

According to the latest data of foreign researchers, due to the influence of heat on the culture of pathogenic Leptospira (temperature 32°C) together with ultraviolet radiation, its accumulation is halved. Therewith, drugs acting with the release of chlorine completely destroy Leptospira during exposure for up to 27 minutes. In addition, the impact assessment was conducted not visually but using cultural and molecular methods [21]. Preservatives such as formaldehyde and paraffin instantly destroy the culture [22].

However, to destroy the antigen of pathogenic cultures of Leptospira, the action of more aggressive factors is necessary. Thus, according to literature sources, it can be inactivated at a temperature of 121°C or using phenol, formalin, or thiomersal heated to 50°C [23].

Additionally, a study was conducted on the effect of disinfectants "Diolide" and "Biolide" on the growth of cultures of pathogenic Leptospira. These crops were placed in an environment with the addition of these drugs in concentrations, and when checking the resistance indicators. As a result, on the 10th day of the experiment, no growth was found in all the investigated cultures. This result is associated with changes in the reaction of the environment (ph) and osmotic pressure in test tubes with culture since leptospirosis pathogens are extremely sensitive to changes in these indicators [24].

Conclusions

The possibility of using complex disinfectants of various chemical natures, which have oxidising properties, for preventive and forced disinfection in leptospirosis was justified. Thus, the drug "Biolide" based on hydrogen peroxide, lactic and super-lactic acids has a bactericidal effect on the culture of pathogenic Leptospira in the form of a 0.55% solution when exposed for 30 minutes at a temperature of 24°C. In addition, it is allowed to use this product at a concentration of 0.185%, provided that the exposure is increased to 60 minutes. It was found that the disinfectant "Diolide" (active ingredients sodium chlorite and sodium chloride) destroys Leptospira when it is used in a dilution of 200 mg/dm^3 (concentration of 0.08% of the active substance) during exposure for 15 minutes at the same temperature. It is possible to apply a lower concentration of 0.02% with an increase in exposure to 30 minutes. It was also discovered that both disinfectants have a pronounced bacteriostatic effect on Leptospira, since they inhibit the growth of these microorganisms even in minimal concentrations.

The next stage of this study will be to examine the possible effect of Biolide and Diolide disinfectants on other types of microbial test cultures.

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Вплив дезінфектантів різної хімічної природи на культуру патогенних лептоспір

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Анотація. Інфекційні хвороби завдають тваринницьким господарствам значних економічних збитків, тому проводиться постійний пошук нових засобів профілактики захворювань, а особливо дезінфектантів. Аналіз наукової літератури вказує на значну проблему лептоспірозу в Україні та фактично відсутні дані щодо застосування комплексних окиснювальних препаратів для його профілактики. Метою роботи було дослідити вплив дезінфектантів біолайд (діючі речовини перекис водню, молочна та надмолочна кислоти) та діолайд (діючі речовини натрію хлорит і натрію хлорид) на збудників лептоспірозу. Для цього, перевіряли стійкість восьми, циркулюючих в Україні, патогенних культур лептоспір різного віку та їхні ростові властивості, шляхом додавання до них різних концентрацій зазначених дезінфектантів. Одержані результати статистично аналізували в програмному забезпеченні Epitools – Epidemiological Calculators. З'ясовано ефективні концентрації та експозицію біолайду та діолайду для застосування при профілактичній та вимушеній дезінфекції при лептоспірозі. В результаті проведених досліджень щодо впливу обох дезінфектантів на 7-ми, 10- та 15-добові тест-культури лептоспір, не було зареєстровано різниці між показниками їхнього накопичення (кількість мікробних клітин/см^3). Тому, одержані результати по культурам різного віку враховували як повторюваність. Обґрунтовано, що для профілактичної та вимушеної дезінфекції при лептоспірозі рекомендовано до використання 0,55 % розчин засобу «Біолайд» за експозиції 30 хв за температури 24 °С. У разі збільшення терміну експозиції до 60 хв, допускається зниження концентрації засобу до 0,185 %. Щодо препарату «Діолайд», то його при цьому зоонозі рекомендовано застосовувати у розведенні 200 мг/л (концентрація 0,08 % за діючою речовиною) впродовж експозиції 15 хв за температури 24 °С. У разі збільшення терміну експозиції до 30 хв, допускається зниження розведення засобу до 50 мг/дм^3 (концентрація 0,02 % за діючою речовиною). До того ж, встановлено, що обидва дезінфікуючі засоби повністю пригнічують ріст патогенних культур лептоспір. Практична цінність роботи полягає у доведенні можливості застосування комплексних дезінфектантів на основі окиснювачів для профілактики лептоспірозу

Ключові слова: ветеринарна медицина, лептоспіроз, дезінфекція, біолайд, діолайд, бактерицидність