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EVALUATION OF VIRULICIDAL EFFECT OF BIOCIDAL AGENT ON THE RESISTANCE OF PARVOVIRUS STRAIN TEST CULTURE

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Abstract. *The paper presents the results of a study of the virucidal efficacy of the biocidal agent "Tetrasept", the main active ingredients of which are glyoxalic aldehyde and glutaric aldehyde. The study was conducted in accordance with national and international guidelines for characterizing the virucidal properties of disinfectants. The toxicity of the biocidal agent "Tetrasept" was studied under conditions of protein loading in a rabbit kidney cell culture model (RK-13). The study of the virucidal efficacy of the biocidal agent "Tetrasept" was carried out under conditions of protein load on the model of the field strain of parvovirus "Antey". Toxicity and virucidal activity of "Tetrasept" were determined for working concentrations of 1.0%, 0.75%, 0.5%, 0.25%, 0.125% and 0.06%, 0.03% at exposure of 30 and 60 minutes, respectively.*

The results of the study showed that the biocidal agent "Tetrasept" is non-toxic to the transplanted cell cultures at concentrations of 0.125%, 0.06% and 0.03%, but has a weakly expressed virucidal effect. The disinfectant has 100% virucidal effect against the field strain of parvovirus at concentrations of 0.25% at exposure for 30 and 60 minutes at protein load, which indicates high virucidal activity of the biocidal agent "Tetrasept". Concentrations of 0.5 %, 0.75 % and 1.0 % have a toxic effect on cell culture.

Key words: virucidal activity, parvovirus strain, biocidal agent, active ingredients, cell culture, toxicity

The field of veterinary medicine is in a state of constant evolution, so the search for new, effective and harmless disinfectants, especially complex ones, has been and remains an urgent problem in the industry. The problem of effective, harmless virucidal agents remains one of the most important tasks of veterinary medicine, so the creation of complex biocidal agents that are safe for humans and animals is a prospect for widespread use. The prevalence of bacterial and viral infections leads to enormous losses every year. At the same time, effective safe means of preventing infectious diseases have not been developed sufficiently. In parallel with the active synthesis of new, more effective bactericidal compounds, it is necessary to test existing products. This testing is multilevel, conducted at several sites, both *in vitro* and *in vivo*.

A significant number of viral infections can be fatal for both animals and humans, especially if the body's immune system is suppressed. For example, viral gastroenteritis

caused by rotaviruses or noroviruses, as well as respiratory viruses such as influenza, parainfluenza, enteroviruses or respiratory syncytial virus can cause large outbreaks of disease and have a rapid spread among susceptible animals. Therefore, disinfection, as the main measure affecting the second link of the epizootic chain, plays an important role in the prevention and control of infectious disease outbreaks on farms (Chechet, 2022; Rabenau, 2014). Certain groups of microorganisms have certain structural features that make them able to resist the effects of disinfectants. For example, enveloped viruses, such as enteroviruses and noroviruses, are particularly resistant to a significant number of biocidal agents, so it is recommended to use only disinfectants with confirmed virucidal activity (Sukhrie, 2010; Scagnolari, 2012). To this end, biocides must be tested for virucidal efficacy in accordance with good laboratory practice and country-specific standards. In Europe, the EN 14476 standard is used to determine virucidal activity, which includes two enveloped viruses and a quantitative suspension assay (EN 14476, n.e.). This quantitative suspension assay is performed in a test tube. But the problem is that the viral particles are suspended in a large volume of biocide, which makes it easier to inactivate the viruses due to the large number of contacts between the disinfectant and the viral particles.

Thus, suspension studies do not reflect the real conditions in production, because in practical conditions, viruses accumulate on surfaces with different protein loads of biological substances that protect viruses from disinfection. Accordingly, it is necessary to prove that the disinfectants on the surfaces are capable of disinfecting microorganisms, so their effectiveness must be tested under conditions close to real life. This type of practical analysis has long been standardized for testing the bactericidal activity of chemical disinfectants (Chechet, 2022; EN 13697, n.e.). Biocidal agents are tested in stages in accordance with European testing principles, starting with a suspension test (EN phase 2, step 1) and then a quantitative test on a non-porous media simulating practical conditions (EN phase 2, step 2) (EN 13697, EN 13727, n.e.)

Currently, there is no standard for testing virucidal efficacy that simulates practical conditions on model viruses that can be dried on media. The main requirements for such viruses are: high resistance to biocides and desiccation combined with easy reproduction of the virus in cell culture. For this purpose, scientists recommend the use of animal parvoviruses, such as the Haden strain of bovine parvovirus (BPV), which is used for disinfection in the quantitative suspension test and the national surface test (Blümel, 2009; Kovalenko, 2021; Rutala, 2019). These viruses are environmentally stable and practical for laboratory use, and safe for workers during the experiment. However, this single-stranded DNA virus requires primary cells to replicate, making it difficult to work in a testing laboratory. Another animal parvovirus, such as canine parvovirus (CPV), can be cultured in continuously growing cells and is similar to bovine parvovirus in terms of stability. Thus, it can be chosen as a model virus for a test.

The aim of the study was to test the effectiveness of the biocidal agent by a virulicidal activity testing on surfaces that simulate natural conditions. The virus was dried on stainless steel disks and exposed to the biocide.

Materials and methods. The object of the study was a sample of the biocidal drug “Tetrasept” produced by the private enterprise “Kronos Agro”, Ukraine. This preparation consists of the following active substances: glyoxalic aldehyde, glutaric aldehyde, benzalkonium chloride, dodecyl dimethylammonium chloride and is a clear, liquid liquid with a characteristic odor of aldehyde. It is highly soluble in water. After laboratory tests, it was found that it has bactericidal, virucidal, and fungicidal properties.

The method of determining the effect of the Tetrasept biocidal solution was performed using animal cell cultures in accordance with the guidelines (Chechet, 2022; SSRILDVSE, 2022).

Methodology of the study. Preparation of a dilution of the disinfectant under test (concentration: 1.0%) in a sterile vial. To do this, take 1.0 cm^3 of disinfectant + 99.0 cm^3 of PSB, mix thoroughly and use in further studies. A 0.5% concentration of disinfectant was also prepared. To do this, we took 0.5 cm^3 of disinfectant + 99.5 cm^3 of PSB, mix thoroughly and use in further studies. After that we prepared 10-fold dilutions of the virus in PSB with each concentration of disinfectant.

To study the effect of the disinfectant on the virus, the following dilutions of solutions were performed: 1.0 cm^3 of the disinfectant at a concentration of 1.0 % was first added to separate penicillin vials (6 pcs.), and then the same vials were labeled accordingly, namely: 1) 10^{-4}+DS 1.0%, 2) 10^{-5}+DS 1.0%, 3) 10^{-6}+DS 1.0%, 4) 10^{-7}+DS 1.0%, 5) 10^{-8}+DS 1.0%, 6) 10^{-9}+DS 1.0%. Subsequently, an equal amount of the appropriate virus dilution was added to the appropriate labeled vial. The components were mixed and left for 30 minutes at room temperature. Thus, the concentration of the disinfectant in each vial was already 0.5%.

Also disinfectant solution dilutions were prepared in separate penicillin vials - 6 pcs. per dilution. We placed 1.0 cm^3 of disinfectant with concentration 0.5% in first dilution, and then the same vials were labeled accordingly, namely: 1) 10^{-4}+DS 0.5%, 2) 10^{-5}+DS 0.5%, 3) 10^{-6}+DS 0.5%, 4) 10^{-7}+DS 0.5%, 5) 10^{-8}+DS 0.5%, 6) 10^{-9}+DS 0.5%. Subsequently, an equal amount of the corresponding virus dilution was added to the appropriate labeled vial. The components were mixed and left for 30 minutes at room temperature. Thus, in each vial, the concentration of disinfectant was already 0.5%, respectively, and the same was done with a concentration of DS of 0.25%, but the exposure was already 60 minutes. The other 1.0 %, 0.75 %, 0.125 %, and 0.06 %, 0.03 % concentrations of the biocide were prepared in the same way.

Estimation of the disinfectant effect on test culture of the field strain of parvovirus “Antey” (certificate of initial deposit of the microorganism strain in the Repository of the State Scientific and Control Institute of Biotechnology and Microorganism Strains (No. 734 of 10.12.2018)). The titer of parvovirus infectivity in rabbit kidney cell culture (RK-13) averaged 1.5 lg $TCID_{50/cm3}$, after the third passage - up to 2.7 ± 0.06 lg $TCID_{50/cm3}$, and after the fifth passage - increased to 3.8 ± 0.08 lg $TCID_{50/cm3}$ (Radzyhovskyi, 2024; Radzikhovskyi, 2022). The use of this type of media for cell cultures not only allows for faster completion of the planned studies, but also minimizes nonspecific factors affecting the course of the experiments.

When using microplates for such studies, there is a possibility of an increase in the error in studies associated with a decrease in the amount of culture fluid and the tested disinfectants in volumes. To compensate for the magnitude of the error, it is advisable to increase the number of replicates (wells of the plate) for the study of one concentration of disinfectant for studies with an expectation of high accuracy.

Contact of the virus-containing suspension with the disinfectant at room temperature recommended by the manufacturer during disinfection for 30 and 60 minutes. Next, sequential procedures were performed, step by step:

1) Application of the mixture (reacted disinfectant with virus) to the monolayer of cell culture, 10 wells for each dilution.

2) Adsorption of the mixture (virus + disinfectant) on the cell culture for 20 minutes.

3) Remove the mixture from the surface of the cell culture monolayer.

4) Washing the monolayer with Hanks' salt buffered saline 3-5 times.

5) Addition of maintenance medium (without animal serum) to the cell culture media.

6) Incubation with daily monitoring of changes in the cell culture monolayer every 12-24 hours.

7) Protocol of the results obtained Observation of the cell culture monolayer was completed after the onset of visible changes in the control wells with cell culture.

In 8 wells with rabbit kidney cell culture, instead of a mixture of disinfectant and viral suspension, a salt buffer was added, and in 8 wells with rabbit kidney cell culture, instead of a mixture of disinfectant and viral suspension, a working dose of virus (10,000 $TCID_{50}$) was

added and considered control wells. Subsequently, the control wells were treated as well as the experimental wells.

The disinfectant effect on the animal virus of the test agent (disinfectant) was expressed in the absence of cytopathic effect in the monolayer of cell culture in certain dilutions of the disinfectant.

Results of the study. After the addition of the biocidal agent “Tetrasept” in working concentrations of 1.0 %, 0.75 %, 0.5 %, 0.25 %, 0.125 % and 0.06 %, 0.03 % to the transplantation line of rabbit kidney cell culture, it was found that the cells in the monolayer remained viable in the presence of the disinfectant in concentrations of 0.25 %, 0.125 % and 0.06 %, 0.03 % (Fig. 1). At the same time, disinfectant concentrations of 0.5 % and above, while showing a virucidal effect on the parvovirus present in the cell culture, also affected the cells of the monolayer due to the influence of the active ingredients of the disinfectant (Fig. 2). The control wells with rabbit kidney cell cultures remained intact during the entire observation period (72 h). In the control wells infected with the working dose of the virus, CPE was observed throughout the monolayer starting from 20 hours after infection (Fig. 4). The results of the study are shown in Table 1.

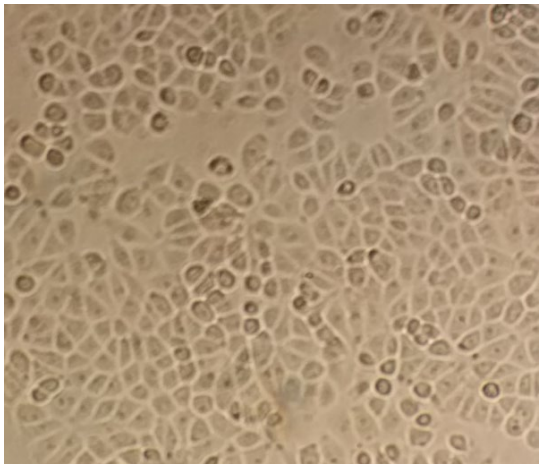

Figure 1. Continuous rabbit kidney cell culture line (RK-13) 12 h after seeding and treatment with a 0.25% disinfectant solution (magnification × 56)

In this case, we considered the study of the biocidal drug toxicity criterion in cell culture, as an alternative to determining toxicity in laboratory animals. Of the test concentrations tested, only 0.03 % solution did not affect the cell culture, but, as we can see, the solution is not effective against parvovirus, which causes damage to the monolayer of cell cultures causing cytopathogenic effects. Therefore, we further considered higher concentrations of the drug in the study.

The biocidal agent “Tetrasept” in concentrations of 0.06 and 0.125 % had a slight toxic effect on the continuous rabbit kidney cell culture line (RK-13), which was recorded in the delay in the formation of a monolayer, which usually had 100 % after 48 h, and under the influence of the above concentrations it was formed by 80 %. Higher concentrations of disinfectant, starting from (Fig. 3). It should also be noted that these concentrations of disinfectant had a slight virucidal effect and, as a result, the effect of the virus with the manifestation of CPE was observed.

Table 1

The effectiveness of disinfectant activity of "Tetrasept" against the field strain of parvovirus "Antey"

Concentration of Tetrasept, % + parvovirus of Antey atrain, 10,000 $TCID_{50}$							Efficacy, % N = 10		Control	
									Cell culture N = 8	Virus, 10,000 $TCID_{50}$ N = 8
1,0	0,75	0,5	0,25	0,125	0,06	0,03	Exposition, min.			
							30	60		
X	X	–	–	#	#	#	100	100	–	#
X	X	X	–	–	#	#	100	100	–	#
X	–	–	–	–	–	#	100	100	–	#
X	X	–	–	–	#	#	100	100	–	#
X	–	–	–	#	–	#	100	100	–	#
X	–	–	–	–	–	#	100	100	–	#
X	–	–	–	–	–	#	100	100	–	#
X	X	–	–	–	#	#	100	100	–	#
X	–	X	–	–	–	#	100	100		
X	–	–	–	–	–	#	100	100		

Note: # - the presence of CPE in rabbit kidney cell culture (RK-13);
X - toxic effect of the biocidal preparation on the cell culture;
- no virus CPE in rabbit kidney (RK-13) cell culture.

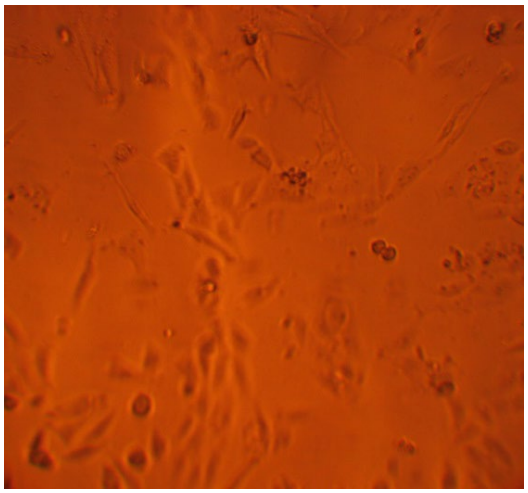

Figure 2. Continuous rabbit kidney cell culture line (RK-13) 72 h after parvovirus infection and 0.5% disinfectant concentration (magnification × 56)

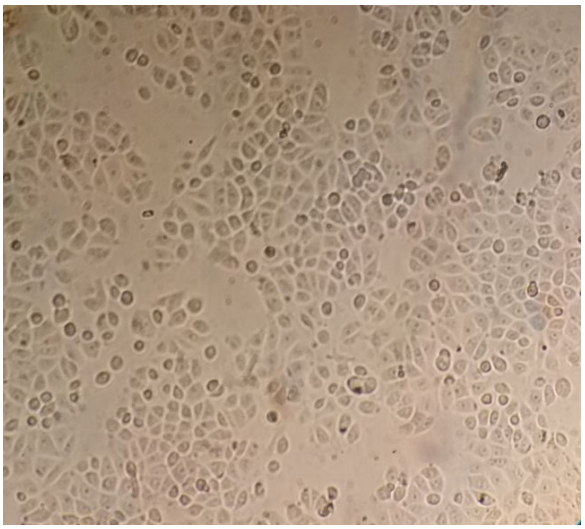

Figure 3. Continuous rabbit kidney cell culture line (RK-13) 48 h after treatment with 0.125% disinfectant solution (magnification × 56)

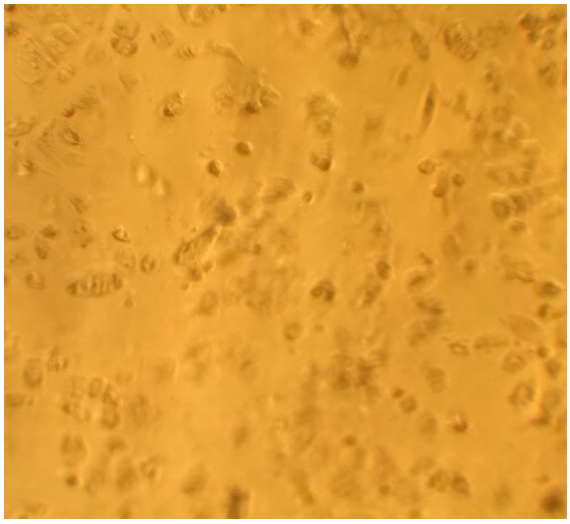

Figure 4. Control wells infected with the working dose of the virus caused CPE throughout the monolayer starting from 20 h after inoculation (magnification × 56)

Thus, the cytotoxic effect of the disinfectant “Tetrasept” in rabbit kidney cell culture at 1.0 % concentration and higher exposures of 30 and 60 min was occured. Thus, in the culture of rabbit kidney cells under the influence of the drug in this concentration after 24 hours of cultivation, a change in morphology and a decrease in the number of living cells and their active proliferation were observed (Fig. 2). The cytotoxic effect of Tetrasept was clearly observed at 48-72 h of cultivation. A 90-100% death rate of cell cultures with no active proliferation of the remaining living cells was detected.

In rabbit kidney cell cultures treated with Tetrasept solution at concentrations of 0.125 % with exposure for 30 and 60 min, no complete cell death was detected during the entire observation period (72 h), respectively. A slight proliferation of cell cultures was observed compared to the control wells.

The results of the study showed that Tetrasept at 0.03 % concentration did not show virucidal activity at exposure of 30 and 60 min under conditions of protein loading. The presence of virus CPE in rabbit kidney cell culture (RK-13) was observed.

Discussion. According to our studies, the main criterion for the initial selection of chemical compounds is the absence of toxic effects on a biological object in working, virucidal, concentrations (Chechet, 2022; Cadnum, 2021). A commonly accepted model for the initial stages of drug bioassay is the culture of differentiated and undifferentiated animal cells. In accordance with the Convention for the Protection of Warm-blooded Animals and from an economic point of view, in vitro methods, by reducing the time required to obtain reproducible and reliable data on the biological activity of a large number of compounds, sometimes using automated measurement systems under different experimental conditions, help to accelerate the development and introduction of new disinfectants (Kindermann, 2020). The common problem of scientists in research to determine the safety and harmlessness of drugs from different active substances prompts the transition to their study using alternative methods, such as tissues and cells, computer modeling, the use of lower forms of organisms (algae, aquatic organisms, plants), etc. Detecting the toxicity of compounds at the early stages of testing reduces the financial costs of studying substances that will not be introduced in the future (Kotsiumbas, 2018; Tarka, 2021). Therefore, the development and introduction of new, environmentally friendly, effective, and animal-friendly complex disinfectants into production is extremely important (Kovalenko, 2013).

Conclusion. Biocidal agent “Tetrasept” has high virucidal activity against canine parvovirus at 0.25% concentration at exposure for 30-60 mins, which allows it to be recommended for disinfection of various livestock facilities.

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ОЦІНКА ВІРУЛІЦИДНОГО ВПЛИВУ БІОЦИДНОГО ЗАСОБУ НА СТІЙКІСТЬ ТЕСТ КУЛЬТУРИ ШТАМУ ПАРВОВІРУСУ

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Резюме. *У статті представлені результати дослідження віруліцидної ефективності біоцидного засобу «Тетрасепт», основними діючими речовинами якого є гліоксалевий альдегід, глутаровий альдегід. Дослідження проводили відповідно до національних і міжнародних керівництв щодо характеристики віруліцидних властивостей дезінфікуючих засобів. Дослідження токсичності біоцидного засобу «Тетрасепт» проводили за умов білкового навантаження на моделі культури клітин нирки кроля (RK-13). Дослідження віруліцидної ефективності біоцидного засобу «Тетрасепт» проводили за умов білкового навантаження на моделі польового штаму парвовірусу «Антей». Токсичність та віруліцидну активність препарату «Тетрасепт» визначали для робочих концентрацій 1,0 %, 0,75 %, 0,5 %, 0,25 %, 0,125 % та 0,06 %, 0,03 % за експозиції 30 та 60 хв відповідно.*

Результати дослідження показали, що біоцидний засіб «Тетрасепт» є нетоксичним для перещеплюваних культур клітин у концентраціях 0,125 %, 0,06 % та 0,03 %, але має слабо виражену віроцидну дію. Дезінфікуючий засіб діє 100 % віруліцидно відносно о польового штаму парвовірусу у концентраціях 0,25 % за експозиції 30 та 60 хв за білкового навантаження, що свідчить про високу віруліцидну активність біоцидного засобу «Тетрасепт». Концентрація 0,5 %, 0,75 % та 1,0 % проявляють токсичну дію на культуру клітин.

Ключові слова: віруліцидна активність, штам парвовірус, біоцидний засіб, діючі речовини, культура клітин, токсичність.

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