Part 3. Biosafety

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VIRUCIDAL ACTIVITY OF DISINFECTANT 'BIOLAID'

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Summary. The article presents the results of the study of toxic and virucidal action of the disinfectant 'Biolaid', which includes hydrogen peroxide, lactic acid, and supralactic acid. The research was conducted following the 'Methodical Approaches to the Control of Disinfectants for Veterinary Medicine' (Kovalenko and Nedosiekov, 2011). Toxicity of the disinfectant 'Biolaid' was characterized in SPEV and BHK-21/C13 cell cultures (ATCC CCL-10). Determination of virucidal activity of the disinfectant 'Biolaid' was performed on models of Aujeszky's disease virus (strain 'Arsky') and rabies virus (strain CVS-11, ATCC VR 959). The toxic effect of the drug 'Biolaid' was determined for concentrations of 2.0%, 1.5%, 1.0%, 0.5%, and 0.25% at exposures of 30 and 60 min in an incubator at 37°C. The virucidal effect of 'Biolaid' was determined for similar concentrations using working dilutions of viral suspensions: for Aujeszky's disease virus — 4.0 CPE_{50}/cm^3, for rabies virus — 4.0 $TCID_{50}/cm^3$. The results of the study showed that the disinfectant 'Biolaid' is not toxic to SPEV and BHK-21/C13 cells in all test concentrations (2.0%, 1.5%, 1.0%, 0.5%, and 0.25%) at exposures of 30 and 60 min. Disinfectant 'Biolaid' has 100% virucidal activity against Aujeszky's disease virus (strain 'Arsky') and rabies virus (strain CVS-11, ATCC VR 959) in all tested concentrations (2.0%, 1.5%, 1.0%, 0.5%, and 0.25%). The virucidal effect of these viruses was manifested at exposures of both 30 and 60 min. The obtained results give grounds to recommend disinfectant 'Biolaid' for disinfection of various livestock and poultry farms in case of detection of viral infections

Keywords: rabies virus, Aujeszky's disease virus, virucidal activity, cell culture

Introduction. The introduction of intensive technologies for the production of livestock products involves a significant concentration of livestock in a limited area, which creates conditions for a significant spread of opportunistic and pathogenic microflora and the emergence of infectious animal diseases. It is necessary to develop new effective methods and means to ensure stable epizootic welfare of livestock. One such method is the use of highly effective disinfectants at all stages of livestock production. The effectiveness of disinfectants should be studied at the stage of their development and selection of substances, because a significant number of disinfectants are toxic, immunosuppressive and cause long-term effects on animals and have high corrosive activity (Curran, Wilkinson and Bradley, 2019; Kovalenko et al., 2018; Paliy et al., 2018). Today, bactericidal preparations based on lactic acid are widely used. They are readily soluble in water, colorless, have high bactericidal and surface activity, combined with low toxicity and the absence of irritants and other side effects (Addie et al., 2015; Van Haute et al., 2015; Ríos-Castillo, González-Rivas and Rodríguez-Jerez, 2017; Cap et al., 2019). They do not form toxic products, are not inactivated by proteins, and are non-aggressive (Kovalenko et al., 2020).

Along with this, there is a need for multilevel testing, including *in vitro* systems. The generally accepted models for biotesting of bactericidal drugs are cultures of differentiated and undifferentiated animal cells. From an economic point of view, *in vitro* methods, reducing the time to obtain reproducible and reliable data, promote the introduction of new disinfectants.

Detection of toxicity of compounds in the early stages of testing reduces the financial cost of studying substances that will not be implemented in the future. In addition, it is possible to test disinfectants for virucidal activity against many viruses using cell cultures, which in experiments *in vivo* requires special biosafety conditions and significant financial costs (Ríos-Castillo, González-Rivas and Rodríguez-Jerez, 2017; Cupo and Beckstead, 2019).

Therefore, the development and implementation of new, environmentally friendly, effective, harmless to animals complex disinfectants is an important scientific field.

Aim of the study. The purpose of the work was to investigate the virucidal activity of disinfectant 'Biolaid' in cell cultures to Aujeszky's disease virus and rabies virus.

Materials and methods. Study of toxicity and virucidal activity of the disinfectant 'Biolaid' were performed following the 'Methodical Approaches to the Control of Disinfectants for Veterinary Medicine' (Kovalenko and Nedosiekov, 2011). 'Biolaid' contains active substances: hydrogen peroxide, lactic acid, and supralactic acid.

Determination of the level of toxic effects of disinfectant 'Biolaid' was performed in continuous cultures of SPEV (pig embryo kidney) and BHK-21/C13 (ATCC CCL-10) cells.

Determination of the virucidal action of the disinfectant 'Biolaid' was performed on models of Aujeszky's disease virus (strain 'Arsky') and rabies virus (strain CVS-11, ATCC VR 959). Infectious activity of Aujeszky's disease virus (strain 'Arsky') 7.31 ± 0.20 lg CPE_{50}/cm^3, rabies virus (strain CVS-11) with infectious activity 7.53 ± 0.11 lg $TCID_{50}/cm^3$.

The following reagents were used for the study: DMEM (Dulbecco's Modified Eagle Medium), Sigma (GB); Fetal Bovine Serum (FBS), Gibco (Brazil); Dulbecco's Phoshate Buffered Saline (DPBS), Sigma (GB); Trypsin-EDTA (0.5%), no phenol red, Gibco (GB); Plasmocin, InvivoGen (France); Antibiotic-Antimycotic, Sigma (Israel); culture microplates (96-well), Sarstedt (Germany); tissue culture flask (75 cm^2), Sarstedt (Germany); ACA acetone, 80%, (Ukraine); FITC Anti-Rabies Globulin Kit, Fujirebio (USA).

The studies were performed using the following equipment: CO_2-incubators Esco Cellulture and Jouan 150; microscope Zeiss — Aviovert 40CFL; inverted luminescent microscope Zeiss AXIOVERT 25CA; Eppendorf and Biohit variable volume dispensers for 20–200 µl and 100–1,000 µl; Jokan MSC9 biosafety cabinets; Holten SAFE-2010 and Hereus HS-18; Naibor's cell counting chamber.

Study of 'Biolaid' toxicity. Plating of SPEV and BHK-21/C13 cell cultures in 96-well microplates (seed concentration of 1.0–1.2×10^5 cells/well) was prepared. After 24 h, the medium was removed from the 96-well microplates (subject to availability of 80–90% monolayer) and appropriate dilutions of disinfectant 0.05 cm^3/well, previously prepared on DMEM medium with 10% FBS at a final concentration of 2.0%, 1.5%, 1.0%, 0.5%, and 0.25% were added.

Contact of SPEV and BHK-21/C13 cells with appropriate disinfectant dilutions was performed in an incubator at 37°C (for BHK-21/C13 cell culture also 5% CO_2) for 30 and 60 min. 32 wells were used for one concentration of 'Biolaid' disinfectant.

For control, SPEV and BHK-21/C13 cells, DMEM medium with the addition of 10% FBS was added to 32 wells of a 96-well microplate, 0.05 cm^3/well for a similar period of time of cell contact with the disinfectant.

At the end of the contact period, disinfectant solutions were removed from the 96-well microplates, they were washed three times with DPBS, and 0.20 cm^3 of maintenance medium containing 10% FBS was added to each well. Incubation of 96-well micropanels with SPEV and BHK-21/C13 cell cultures was performed for 72 h with daily microscopy of the cell monolayer in the wells for cytopathic effect (CPE).

'Biolaid' virucidal studies. The disinfectant effect of 'Biolaid' was determined for concentrations of 2.0%, 1.5%, 1.0%, 0.5%, and 0.25%. Test items: Aujeszky's disease virus (strain 'Arsky') and rabies virus (strain CVS-11, ATCC VR 959). Preliminarily, cultures of SPEV and BHK-21/C13 cells were plated in 96-well microplates (plating concentration of 1.0–1.2×10^5 cells/well).

In each experiment, the working dilution of viral suspensions was obtained on the basis of virus activity titers: for Aujeszky's disease virus — 4.0 CPE_{50}/cm^3, for rabies virus — 4.0 $TCID_{50}/cm^3$. A certain amount of disinfectant was added to the viral suspensions to obtain the appropriate final concentration: 2.0%, 1.5%, 1.0%, 0.5%, and 0.25%.

Contact of viral suspensions with appropriate dilutions of disinfectant was performed at room temperature (recommended by the manufacturer for disinfection) for 30 and 60 min. 32 wells with SPEV and BHK-21/C13 cell cultures were used for each concentration of 'Biolaid' disinfectant.

After that, appropriate dilutions of disinfectant with Aujeszky's disease virus (strain 'Arsky') were added to the daily monolayer of SPEV cell cultures and dilutions of disinfectant with rabies virus (strain CVS-11, ATCC VR 959) were added to the daily monolayer of BHK-21/C13 cell cultures. Adsorption of the mixture of virus and disinfectant in cell cultures for 30 and 60 min.

Dilution of virus disinfectant was then removed from the 96-well micropanels, washed three times with DPBS, and 0.20 cm^3 of maintenance medium containing 10% FBS was added to each well.

96-well micropanels with SPEV cell culture containing different concentrations of disinfectant and working dilution of Aujeszky's disease virus (strain 'Arsky') were incubated for 72 h with daily microscopy of the cell monolayer in the wells for the detection of cytopathic effect (CPE).

96-well micropanels with BHK-21/C13 cell culture containing various concentrations of disinfectant and working dilution of rabies virus (strain CVS-11, ATCC VR 959) were incubated for 72 h. At the end of the incubation period, the cells were fixed in the wells with 80% acetone and, after drying, stained with FITC Anti-Rabies Globulin Kit.

After washing the cells with DPBS, the presence of a specific rabies virus glow was assessed under a fluorescent microscope.

To control the cells we used DMEM medium with the addition of 10% FBS, which was introduced in 32 wells of a 96-well microplate for a similar period of time of adsorption of a mixture of virus and disinfectant. As positive controls, viral suspensions in working dilution (Aujeszky's disease virus — 4.0 lg CPE_{50}/0.2 cm^3, rabies virus — 4.0 lg $TCID_{50}$/0.2 cm^3) were used, which were added to 32 wells of a 96-well microplate.

The disinfecting effect of 'Biolaid' on experimental viruses was expressed in the absence of virus expression in cell cultures, namely: lack of CPE in SPEV cell culture for Aujeszky's disease virus and lack of specific glow of rabies virus in BHK-21/C13 cell culture.

Results and discussion. ***Toxicity study of the drug 'Biolaid'.*** In experiments to determine the toxicity of

'Biolaid' disinfectant, a certain difference in the effect of different concentrations of the drug in cell cultures SPEV and BHK-21/C13 has been determined. In cell cultures SPEV and BHK-21/C13, on the daily monolayer of which disinfectant 'Biolaid' was applied in concentrations of 1.5%, 1.0%, 0.5%, and 0.25%, both for 30 min and for 60 min contact, visually no adverse effects were detected.

The concentration of 2.0% 'Biolaid' disinfectant did not visually have a negative effect on SPEV cell culture. However, the use of this concentration of disinfectant in BHK-21/C13 cell culture reduced the rate of cell proliferation compared to control.

That is, during 24 h of incubation, the monolayer of BHK-21/C13 cells was unchanged at 80–90% at 100% monolayer in wells with cell control. During the next 48 h of cultivation, cell proliferation was restored. At the end of the cultivation period, the monolayer of BHK-21/C13 cells was 100% without signs of adverse effects of disinfectant (compared to control).

Study of virucidal action of the drug 'Biolaid'. Studies of the virucidal activity of the disinfectant 'Biolaid' in the model of Aujeszky's disease virus (strain 'Arsky') in SPEV cell culture showed that all applied concentrations of disinfectant (2.0%, 1.5%, 1.0%, 0.5%, and 0.25%) for 30 min (duration of exposure) showed 100% disinfecting effect (Table 1).

No CPE was detected in any wells containing mixtures of different concentrations of disinfectant and working dilution of Aujeszky's disease virus (strain 'Arsky').

Table 1 — Virulicidal activity of disinfectant 'Biolaid' against Aujeszky's disease virus (strain 'Arsky') in SPEV cell culture

'Biolaid' concentration, %	Exposition, min	Virus presence	Cell control	Virus control (presence of CPE in 24-48-72 h of cultivation)
2.0	30	–	#	+
	60	–	#	+
1.5	30	–	#	+
	60	–	#	+
1.0	30	–	#	+
	60	–	#	+
0.5	30	–	#	+
	60	–	#	+
0.25	30	–	#	+
	60	–	#	+

Notes: '–' — no CPE in cell culture; '+' — the presence of CPE in cell culture; '#' — the presence of 100% monolayer for 72 h of cultivation.

Control wells with SPEV cell culture (Fig. 1a) remained intact throughout the observation period (72 h). In virus-controlled wells (Fig. 1b), 100% CPE was observed as early as 24 h after infection.

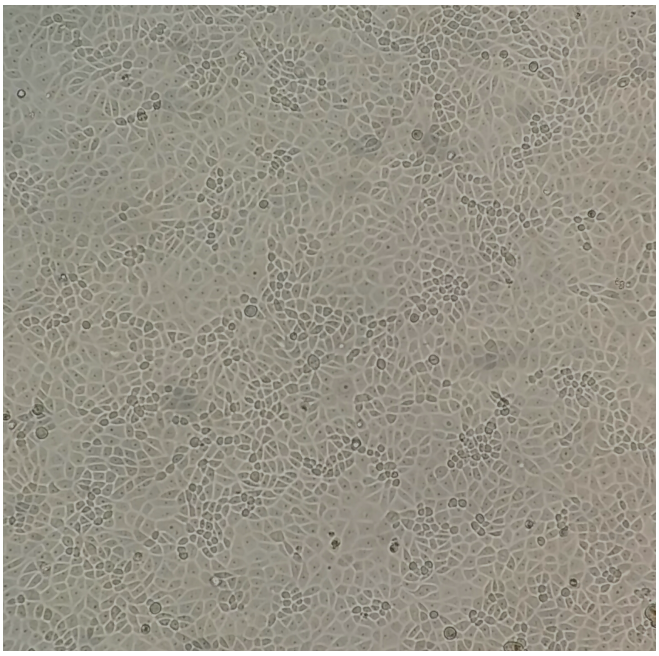

Figure 1a. SPEV cell culture

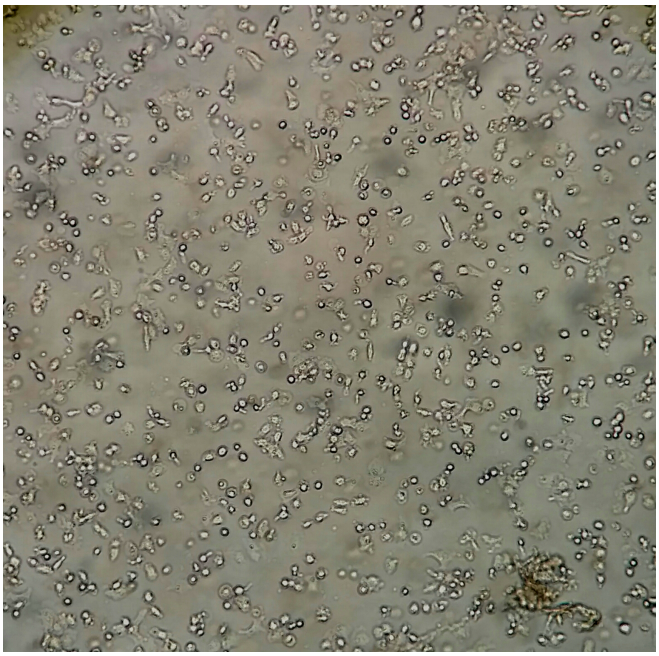

Figure. 1b. Virus control. CPE in SPEV cell culture 24 h after infection with Aujeszky's disease virus (strain 'Arsky')

The infectious titer of the working dose of Aujeszky's disease virus (strain 'Arsky') used in the experiments was 4.22 ± 0.15 lg $CPE_{50}/0.02$ cm^3 at 30 min exposure and 4.37 ± 0.15 lg $CPE_{50}/0.02$ cm^3 for 60 min of exposure.

Studies of the virucidal activity of the disinfectant 'Biolaid' on the model of rabies virus (strain CVS-11, ATCC VR 959) in the cell culture BHK-21/C13 similarly showed that all applied concentrations of disinfectant (2.0%, 1.5%, 1.0%, 0.5%, and 0.25%) for 30 min (duration of exposure) showed 100% disinfectant effect (Table 2).

Table 2 — Virulicidal activity of disinfectant 'Biolaid' against rabies virus (strain CVS-11, ATCC VR 959) in the cell culture BHK-21/C13

'Biolaid' concentration, %	Exposition, min	Virus presence	Cell control	Virus control (presence of a specific glow on the 72nd h of cultivation)
2.0	30	–	#	+
	60	–	#	+
1.5	30	–	#	+
	60	–	#	+
1.0	30	–	#	+
	60	–	#	+
0.5	30	–	#	+
	60	–	#	+
0.25	30	–	#	+
	60	–	#	+

Notes: '–' — the absence of specific glow in immunofluorescence microscopy of cell culture after 72 h of incubation; '+' — the presence of a specific glow of rabies virus in immunofluorescence microscopy of cell culture after 72 h of incubation; '#' — the presence of 100% monolayer after 72 h of cultivation.

No specific glow, characteristic of rabies virus, was detected by fluorescence microscopy in any of the wells, in which mixtures of different concentrations of disinfectant and working dilution of rabies virus were applied, after 72 h of cultivation. In control wells with BHK-21/C13 cell culture (Fig. 2a), the monolayer was 100% during the entire observation period (72 h). In the wells with virus control after 72 h, a specific glow of rabies virus was detected (Fig. 2b).

Titration of the working dose of rabies virus (strain CVS-11, ATCC VR 959) used in the experiments showed a value of 4.75 ± 0.22 lg CPE_{50}/0.02 cm^3 at exposure for 30 min and 4.82 ± 0.15 lg CPE_{50}/0.02 cm^3 at exposure for 60 min.

The results of our studies generally coincide with the information provided by other authors who studied and analyzed experimental data on the toxicity and disinfectant effects of drugs containing hydrogen peroxide, lactic acid and supralactic acid (Goyal et al., 2014; Thomas et al., 2020; Melo et al., 2020; Wlazlo et al., 2020).

Conclusions. 'Biolaid' disinfectant is not toxic to SPEV and BHK-21/C13 continuous cells in concentrations from 2.0% to 0.25%.

'Biolaid' disinfectant has high virucidal activity against Aujeszky's disease virus (strain 'Arsky') and rabies virus (strain CVS-11, ATCC VR 959) in concentrations from 2.0% to 0.25% at exposure for 30–60 min, which allows to recommend it for disinfection of various objects of livestock and poultry farms in case of detection of viral infections, disinfection of veterinary tools and equipment.

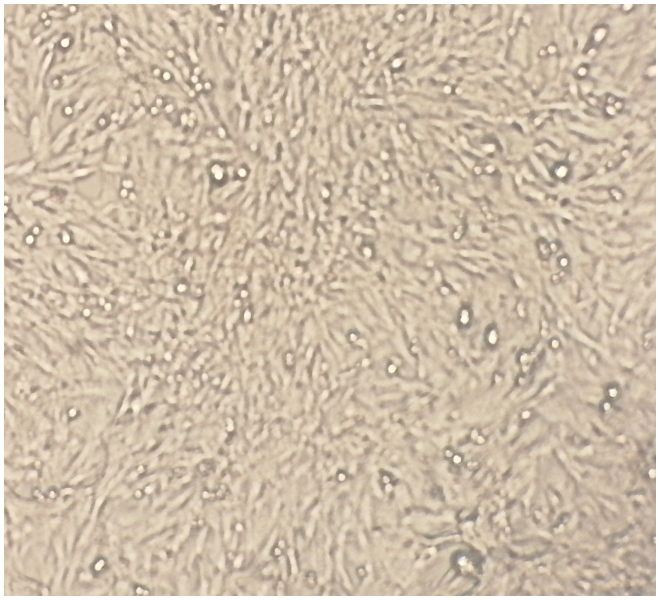

Figure 2a. BHK-21/C13 cell culture

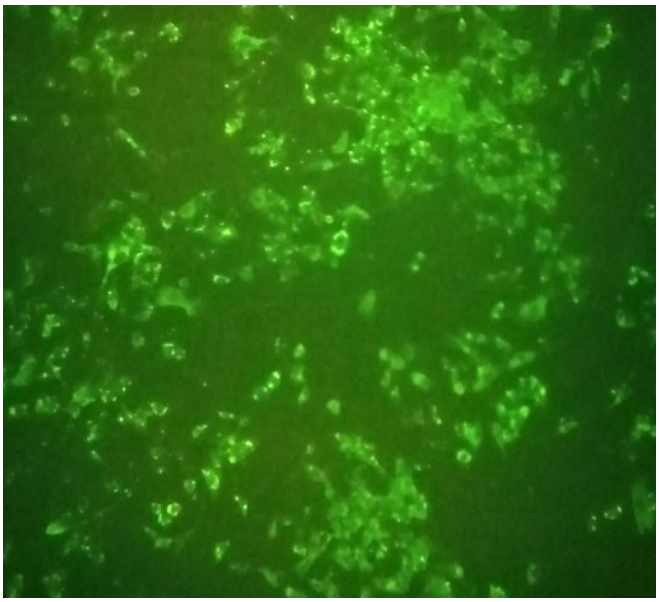

Figure 2b. Virus control. Luminescent microscopy of rabies virus (strain CVS-11, ATCC VR 959) in cell culture BHK-21/C13, 72 h after infection

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