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## REPORT

On the analysis conducted under Contract No. /4 dated 11.12.2025

### INVESTIGATION OF THE VIRUCIDAL ACTIVITY OF THE PRODUCT **Bibiotic® Immuno AGAINST Influenza Virus (IV) AND Coronavirus (HCoV) IN CELL CULTURES**

#### **Samples – Bibiotic® Immuno**

The samples were kindly provided for analysis by "EKOKOM GROUP" JSC in the form of sealed vials containing an opaque dark-brown liquid with a characteristic odor.

The samples were applied in their original form (a new vial was used for each replicate), without filtration, dilution, or any other prior treatment during the virucidal activity assessment. At the beginning of each experiment, they were mixed ex tempore in a 1:1 ratio with a viral suspension.

#### **Cell Cultures**

- **MDCK (Madin–Darby canine kidney)** epithelial cells – ATCC (American Type Culture Collection), USA – used for evaluation of the effect against Influenza A viruses.
- **Vero E6** – kidney epithelial cells derived from African green monkey (*Chlorocebus sabaeus*) (ATCC, CRL-1586), used for cultivation of human coronavirus OC-43 (HCoV-OC43) (ATCC: VR-1558) (Manassas, Virginia, USA).

Cells were cultured at 37°C in a 5% CO<sub>2</sub> incubator (Thermo Forma 310, Thermo Fisher Scientific, MA, USA) as monolayer cultures in T75 polystyrene flasks and 96-well plates (Corning® Costar®, USA), using DMEM growth medium

supplemented with 10% fetal bovine serum (Gibco), 3.7 mg/ml sodium bicarbonate, 10  $\mu$ M HEPES buffer (AppliChem GmbH, Darmstadt, Germany), 100 U/ml Penicillin, 100  $\mu$ g/ml Streptomycin, and 25  $\mu$ g/ml Amphotericin B.

Following thawing, cells were passaged every 72 hours upon reaching 80–90% confluence by treatment with trypsin-EDTA solution (0.05% trypsin, 0.53 mM EDTA). Cell counts were determined using a Bürker chamber and cells were resuspended in fresh growth medium.

Experiments to determine virucidal activity were performed in 24-hour cell cultures seeded at a density of  $2.5 \times 10^5$ /ml in 96-well plates at a minimum of 90% monolayer confluence. Visual and colorimetric assessments were conducted 72–120 hours post-treatment.

### **Viruses**

- Influenza virus A/Panama/2000/99 (H3N2)
- Influenza virus A/Puerto Rico/8/34 (H1N1)  
(kindly provided by the Institute of Virology, NCPHA, Sofia)
- Human beta coronavirus OC-43 (HCoV-OC43) (ATCC: VR-1558) (Manassas, Virginia, USA)

### **Determination of Effect on Extracellular Virions – Virucidal Effect**

A sample containing 1000 CCID<sub>50</sub> of virus was placed in contact with the test preparation (BB) at a 1:1 ratio and incubated at room temperature for varying time intervals (15, 30, and 60 minutes).

Subsequently, samples were titrated onto a 24-hour cell monolayer, and the residual infectious virus content was evaluated 72–120 hours post-infection by assessing cytopathic effect (CPE) using the endpoint dilution method and by staining with the vital dye Neutral Red (NR).

Plates were washed with 150  $\mu$ l PBS without Ca<sup>2+</sup> and Mg<sup>2+</sup> per well, and 100  $\mu$ l of pre-prepared Neutral Red dye solution was added to each well. Stained samples were incubated at 37°C in 5% CO<sub>2</sub> for 3 hours. After incubation, the staining solution was discarded and each well was washed again with 150  $\mu$ l PBS. Immediately prior to removal of the saline solution, an extraction solution consisting of 1% glacial acetic acid (CH<sub>3</sub>COOH), 50% ethanol (C<sub>2</sub>H<sub>5</sub>OH), and 49% distilled water (dH<sub>2</sub>O) was prepared and added at 100  $\mu$ l per well.

Optical density was measured at 540 nm using a microplate reader (Biotek Organon, West Chester, PA, USA). Results expressed in  $\Delta$ log values were

calculated in comparison with viral controls incubated for the same time intervals without the presence of the tested products.

## Statistical Analysis

Experiments were conducted in duplicate and triplicate to ensure statistical reliability. Data were processed using Gen5® and Microsoft Excel®, and graphical representations were generated using GraphPad Prism 9.0®.

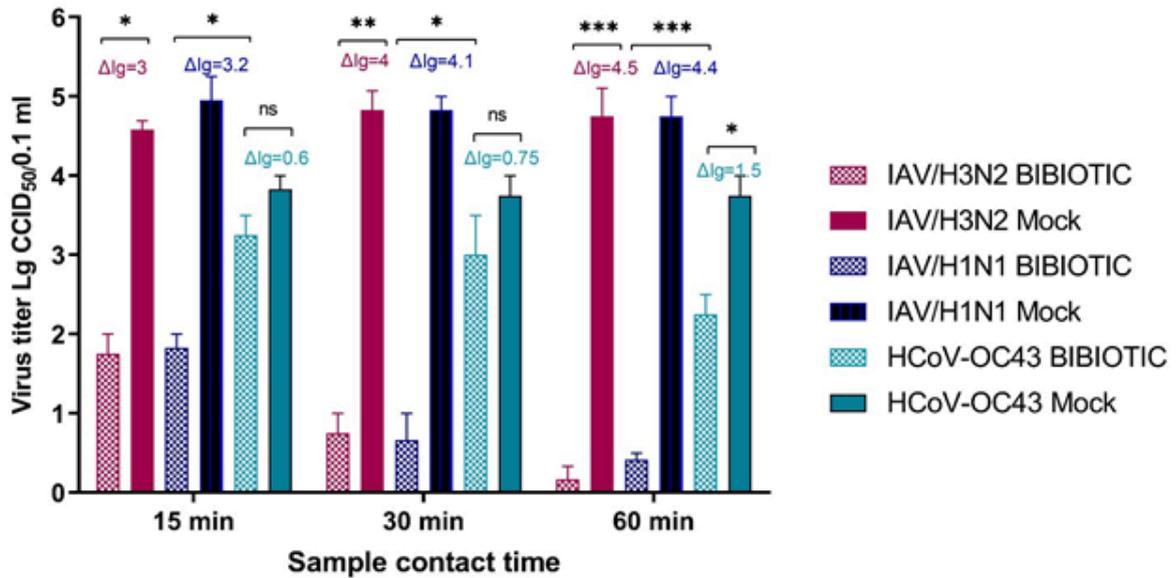
## Results

### 1. Determination of Effect on Extracellular Virions – Virucidal Effect

**Table 1. Virucidal activity in experimental infection with 1000 CCID<sub>50</sub>/ml\***

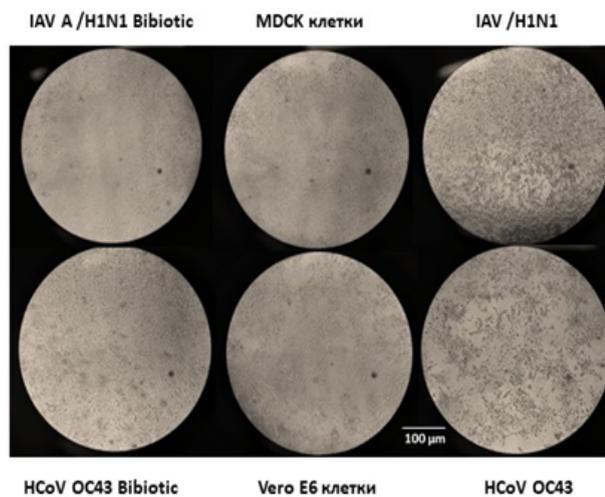
Sample / Virus	Concentration	ΔLg		
		15 min	30 min	60 min
Bibiotic / IAV H3N2	50%	3.0	4.0	4.5
Bibiotic / IAV H1N1	50%	3.2	4.1	4.4
Bibiotic / HCoV OC43	50%	0.6	0.75	1.5
Ethanol (reference) IAVH3N2/IAVH1N1/HCoV	70%	5.0 / 5.0 / 4.0	5.0 / 5.0 / 4.0	5.0 / 5.0 / 4.0

\*Mean values from two independent experiments.



**Statistical analysis:** Error bars: SD – standard deviations; Two-way ANOVA (Bibiotic vs Mock).

**Figure 1.** Virucidal activity of Bibiotic® Immuno against A/Panama/00/99 (H3N2), A/PR/8/34 (H1N1), and HCoV OC43 at contact intervals of 15, 30, and 60 minutes.



**Fig. 1.** Microscopic visualization of the virucidal effect of Bibiotic® Immuno against Influenza A/H1N1 in MDCK cells and HCoV OC-43 in Vero E6 cells after a 60-minute contact interval (Inverted light microscope Olympus CK40, magnification  $\times 10$ ).

## Conclusions

1. The investigation of virucidal activity demonstrated a pronounced effect of Bibiotic® Immuno on extracellular particles of Influenza virus A/Panama/00/99 (H3N2) at virion–sample contact intervals ranging from 15 to 60 minutes ( $\Delta\log=3.0$  /15 min;  $\Delta\log=4.0$  /30 min;  $\Delta\log=4.5$  /60 min/).
2. The investigation of virucidal activity demonstrated a strongly expressed effect of Bibiotic® Immuno on extracellular particles of Influenza virus A/PR/8/34 (H1N1) at virion–sample contact intervals ranging from 15 to 60 minutes ( $\Delta\log=3.2$  /15 min;  $\Delta\log=4.1$  /30 min;  $\Delta\log=4.4$  /60 min/).
3. The investigation of virucidal activity demonstrated a moderate effect of Bibiotic® Immuno on extracellular particles of human coronavirus HCoV OC43 at virion–sample contact intervals ranging from 15 to 60 minutes ( $\Delta\log=0.6$  /15 min;  $\Delta\log=0.75$  /30 min;  $\Delta\log=1.5$  /60 min/).

The study was conducted under BSL-2 biosafety conditions at the Laboratory of Experimental Chemotherapy of Influenza, Department of Virology, Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences.

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