

## ‘NANOVIROSAN’ ANTIMICROBIAL COMPOSITE, DESIGNED FOR EMERGENCY EPIZOOTIC SITUATIONS AND SAFE USAGE IN ECOLOGICAL PIG FARMING

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**Summary.** Analytical data of preclinical and clinical trials of the experimental veterinary composite drug ‘NanoViroSan’ (containing Methisazone, Silgeran and magnesium nanooxide) on laboratory models of Aujeszky’s (AD) and Teschen (TD) diseases, circovirus infections (PCV-2) and actinobacillary pleuropneumonia (APP) as well as in enzootic foci of mixed infection of AD-PCV-APP and swine pox, are presented, respectively. At the level of statistical probability  $p \leq 0.01-0.03$  ( $n = 88$ ), the absence of cytotoxic ( $n = 40$ , cultures of pig testicle cells and pig alveolar macrophages) and biotoxic effects ( $n = 48$  guinea pigs) was proven, as well as high antimicrobial (viro- and bacteriostatic) activity of the drug in the concentration range (by Methisazone) of 1.0–4.0 mg/cm<sup>3</sup>. Intramuscular administration of the drug to male pedigree piglets in doses of 0.5 cm<sup>3</sup>/20 kg and 1.5 cm<sup>3</sup>/20 kg three times with an interval of a day made it possible to stop the carriage of the causative agents of mixed infection in the conditions of pig-breeding ( $n = 26$ ,  $p \leq 0.02$ ). Similar treatment with the drug in a dose of 2 cm<sup>3</sup>/20 kg (by Methisazone) of a boar and five sows in another commodity farm made it possible to break the chain of vertical transmission of the causative agent of swine pox from the nucleus to offspring of the herd ( $n = 227$ ,  $p \leq 0.03$ ). There conclusion was made regarding the perspective of experimental drug for bioprotection of pig farming in the conditions of martial law, as well, if additional research will be positive — as for the development of permaculture (‘green technologies’) in the field of pig breeding

**Keywords:** bioprotection, porcine virobacterial infections, swine pox, permaculture

**Introduction.** Under the current state of the world economy, agricultural export is becoming an increasingly popular and profitable business ([The World Bank, 2022](#); [De Zeeuw and Klank, 1997](#)), and the war of the Russian Federation has highlighted the crucial role that Ukrainian agricultural exports play in ensuring global food security ([FAO, 2022](#)).

Quarantine restrictions on cross-border infections — African swine fever (ASF), foot-and-mouth disease (FMD), Aujeszky’s disease (AD), etc. ([RIRD, 2008](#)) and in addition microbial and antibiotic contamination of pig carcasses, pork, and reproductive materials are well-known problems in the export of pig products ([Monger et al., 2021](#); [APHA, 2022](#)).

In the system of biological safety of industrial pig farms, the most important objects of control are undoubtedly boars’ semen, as well as rearing young animals. After all, it is through them not only the genetic material is exchanged between different pig farms, but also infectious agents — sometimes even the ASF virus ([Schulze et al., 2015](#); [Maes et al., 2008](#); [Gallardo et al., 2015](#)). In this sense, the weakly virulent variants of these pathogens pose the greatest danger. Breeding boars, due to the most powerful constitutional endurance compared to all other technological groups of pigs, usually, as if not the last of the herd, show clinical signs of the disease

([Müller and Brem, 1991](#); [Henryon et al, 2003](#)). Therefore, they often become a source of long-term persistent infection of industrial herds in the form of latent carriers ([Maroto Martín et al., 2010](#); [ANSES, 2018](#)). This is very dangerous for agricultural exports: although the pathogen is ‘weak’, but with further passage in the herd, sooner or later it becomes the trigger of an epizootic outbreak, if it’s not neutralized in the nucleus of the herd — in a group of boars-breeders/their ejaculates as well in repair stock, primarily ([Monga and Roberts, 1994](#)). Moreover, it is known that the contamination of boar semen by *Escherichia coli* can even lead to auto agglutination of spermatozoa and, as a result, to the infertility of sows ([Buzun et al., 2022](#)).

Earlier, we published data on the effectiveness of the first two experimental drugs ‘SuiViroSan’ for the rehabilitation of breeding boars and their sperm from the status of carriers of pathogenic microflora ([Eggers et al., 2021](#)).

The purpose of this article is to analyze the data of our trials of a medicament of this line with more wide antimicrobial range — of the composite veterinary drug (CVD) ‘NanoViroSan’ with activity against the swine pox virus (SPV), porcine circovirus type 2 (PCV-2), *Actinobacillus pleuropneumonia* (APP) and especially against Aujeszky’s disease virus (ADV) and Teschen

disease virus (TDV) which are models of ASF and FMD pathogens, respectively (Tarka and Nitsch-Osuch, 2021; WHO Expert Committee on Specifications for Pharmaceutical Preparations, 2002).

Material and methods. *Test substance and reference drugs.* CVD ‘NanoViroSan’ (batch: 98/27-01-2021, total size 27,540 doses or 12.7 l) is a composite veterinary medicine (Table 1), which includes manufactured in

Ukraine veterinary drugs ‘Methisazone’ and ‘Silgeran’, as well as integrative additives with magnesium oxide nanoparticles enhancing their effect (patent pending). The quality of the composite pharmaceuticals was guaranteed by the relevant Ukrainian technical specifications (UkrTS/UkrNDNC) corresponding with requirements of the Good practices for national pharmaceutical control laboratories (SCVMU, 2007).

Table 1 — Characteristics of the ingredients of the ‘NanoViroSan-L/O’ veterinary drug

No.	Composite components and their functions		
	Composite components	Veterinary drug or substance / their function	Source
1	Methisazone	Methisazone (N-methyl-isatin- $\beta$ -thiosemicarbazone) TU U/UkrNDNC 20.4-25945042270-001:2021, a yellow powder insoluble in water, has a pronounced antiviral effect in vitro in doses that are 100–1,000 times lower than toxic. It inhibits the reproduction of both DNA- and RNA-containing viruses (adenoviruses, herpesviruses, poxviruses, paramyxoviruses, influenza viruses of types A and B, retroviruses, etc.)/Antiviral active substance	Laboratory of Immunology of the Institute of Animal Biology NAAS (Lviv, Ukraine)
2	‘Silgeran-W’	‘Silgeran-W’ (TU U/UkrNDNC 10.9-2661009934-001:2016), Natural complexes of glycooligopeptides and lipopolysaccharides of bacterial origin, mixed with supramolecular complexes of zinc and selenium, citrate and sodium formiate; destroys biofilms of pathogenic microorganisms and prevents their formation, enhances the effect of antibiotics, prevents the chronicity of infections, has pronounced antiviral properties against RNA-containing viruses/Antibacterial active substance	SPC ‘Ariadna’ Ltd. (Odesa, Ukraine)
3	Magnesium Oxide (MgO) Nanoparticles	MgO nanoparticles has a pronounced antiviral effect in vitro in doses that are 1,000 times lower than toxic, help to destroy biofilms of pathogenic microorganisms/Enhancer of composite’s antimicrobial activity (patent application)	NPU ‘KhPI’ (Kharkiv, Ukraine)
4	Vaccine oil	Commercial vaccine oil which is emulsified by hand shaking/prolonging substance (patent application)	Kyiv, Ukraine

They however unlikely encompass the full scope of pharmacological active moieties. Numerous clinical studies have proven the efficacy of these composites in treating swine with different viral and bacterial infections. It is recommended in clinical guidelines (reviewed in approved info of manufacturers).

*Infection agents and their maintenance.* There, the strains of infectious agents that are relevant for Ukraine, isolated from pigs of commodity farms (all pathogens, with the exception of *Teschovirus*) and the domestic sector (*Teschovirus*) were used in the work. Aujeszky’s disease virus (ADV; strain 1082, epizootic, isolate in Donetsk Region on 12.03.2011), Teschen disease virus (TDV; strain ‘Butcha’, epizootic, isolate in Kyiv Region on 21.09.2004), porcine circovirus type 2 (PCV-2, strain ‘1-1024’; epizootic, isolate in Kherson Region on 23.04.2010) and *Actinobacillus pleuropneumonia* type 8 (APP; strain ‘Okhtirka-1411’; epizootic, isolate in Sumy Region on 07.11.2016) were obtained from the Museum of Strains of Microorganisms of the NSC ‘IECVM’ (National Property of Ukraine, Kharkiv, Ukraine). These microorganisms were identified and validated according

to current requirements (SPOU and NASU, 1995), which correspond to international standards (WIPO, 1980). The specified strains are maintained according to the relevant passport data and Standard Operating Procedures of the NSC ‘IECVM’. For testing, under appropriate biosafety conditions, their biological diversity was restored by passage through the organism of a guinea pig (causing agents of AD, TD, and APP) or suckling pigs (PCV-2). After that, the infectious potential of the strains was restored by three consecutive passages through monolayer subcultures of primary swine testicle (ST) cells/testicle cells of suckling piglets (for AD and TD viruses) or porcine alveolar macrophages (PAM, for PCV-2) to titers 6.5–7.5 lgID<sub>50</sub>/ml. (NSC ‘IECVM’, 2020). The tubes’ subcultures of ST cells were growth in Hanks BSS with conditioning by 0.01% yeast extract, 5–7% fetal calf serum (FCS, both Serva), and 0.5% lactalbumin hydrolysate (Sigma-Aldrich), 100 U/ml of penicillin, and 100 g/ml of streptomycin (Brovafarm Co, Kyiv), HIL-FCS. Mature cell monolayer ST subcultures and PAM were maintained with HIL without FCS and yeast extract under a temperature of 37 °C. Test bacteria

maintenance was conducted as recommended by the EU reference center (Sidoli and Pascucci, 1998). To obtain *Actinobacillus pleuropneumonia* test cultures in tubes, its museum suspension after pass per guinea pig organism (see above) was streaked on Petri's dish with chocolate agar supplemented with 1% blood-agar and incubated 24 hours at 37 °C. A suspension of daily bacterial culture was seeded on skew chocolate agar in test tubes (see below).

Studies of antimicrobial properties of the drug were conducted by Ten-concentration Dose Response Assay (TDRA) and Toxicity Assays (TA) taking into account the phenomenon of the dose responses hormesis (Calabrese, 2008) — *in vitro* and *in vivo*.

Briefly, for *in vitro* trials the approximately 20% of tested batch of the CVD 'NanoViroSan' (five random selected flaks) was separated into liquid and oil phases by low-speed centrifugation and liquid phase sterilized by microfiltration per disposable syringe cartridge with 0.3 µm-pores (Argos Technologies, India). In all these experiments, the liquid phase was used: ten concentrations were prepared from it in appropriate solvents (Table 3), which were also used as a blank (solvent control). For studies of virostatic activity cultural cells (ST or PAM) were seeded into cultural tubes (four ones on each drug dilution plus blank and mock cells without drug). After 2 hours of incubation at the 37 °C, ten different concentrations of the drug were added to each tube. ST cells and PAM were then infected with AD or TD viruses at MOI of 0.01 and with PCV-2 at MOI of 0.05, respectively. Mock (cells only with HIL-FCS) and Blank (cells with drug solvent in HIL-FCS) were viewed as positive and virus-infected cells only (HIL-FCS without drug and solvent) count as negative controls, respectively. Viral load was measured by Reed and Muench procedure: by cytopathic effects of the AD and TD viruses or the PCV-2 antigens presence by reaction of passive hemagglutinating (PHA), as prescribed in SOP of the NSC 'IECVM'. Compound cytotoxicity assay were carried out in the same fashion, except without viral infections of cell cultures. Bacteriostatic studies were conducted by analogous schedule, but instead of the cell cultures bacteriological mediums and reference bacteria by SOP of the NSC 'IECVM' were used. Namely, we used the APP agent in the final concentration of 4.5 lg

bacterial cells, BC (by McFarland Standard, HiMedia Laboratories, India), and the bacteriological medium mentioned above. The total bacteria count (i.e. their viable concentration, TVC) of a sample was counted by the number of colony-forming bacterial units (CFU) in 1 ml sample at plate seed.

For *in vivo* trials the same control flaks were used, approximately 20% of the tested batch of the CVD 'NanoViroSan-L/O' (united five random selected flaks before separation into liquid and oil phases as above) and guinea pigs (weight 150–180 g each, four animals in each from three experimental groups — see Table 4).

On the 1<sup>st</sup> day of trials, animals of experimental group 2 were infected with agent AD: 1.2–1.5 cm<sup>3</sup> of the cultural suspension of AD virus (~ 3.0 lg TCID<sub>50</sub>) in the lips of each animal of this group. Guinea pigs of group 3 were infected with agent APP: 1.2–1.5 cm<sup>3</sup> of the suspension of the agar culture of *A. pleuropneumonia* (~ 25,000 BC by McFarland's standard) intraperitoneally to each animal of this group. On the 2<sup>nd</sup> day of trials, there were three intramuscular doses (0.25, 0.50, and 1.50 ml, all injections in hind legs) and, as parallel, the untreated control was applied in all instances also. The course of treatment with the drug included the injections one the same dose three times with an interval of 48 hours in all instances. The results were recorded according to clinical signs with confirmation of the diagnosis by virological or bacteriological studies, respectively, according to the SOP of the NSC 'IECVM'.

*Field tests.* Groups of pedigree male piglets (Great White and Landrace breeds, age 3.5–4.2 months, n = 27) were kept in separate group cages (No. 1–5, see Table 2) of the sanitary facility of the experimental farm of the National Academy of Sciences of Ukraine. Each piglet was fed twice a day with 0.5–0.7 kg of complete grower feed with a protein content of 17–19%. In this group, serological and bacteriological studies, even before the introduction of the clinical protocol, showed asymptomatic carriage of the consortium of causative agents of circovirus infection (PCV-2), Aujeszky's disease (AD) and actinobacillary pleuropneumonia (APP). The clinical protocol of trials aimed to optimize the procedure for using the experimental drug 'NanoViroSan-L/O' according to Table 2.

Table 2 — Doses and schedule of 'NanoViroSan L/O' injections

Time, days	Group cages (experimental groups of pedigree male piglets)				
	1 (n = 5)	2 (n = 5)	3 (n = 7)	4 (n = 5)	5 (n = 5)
0	Forming of experimental groups by breed. Sampling.				
1–3	Subcutaneous injection of 0.5 ml three times a day	Subcutaneous injection of 0.5 ml once a day, three days in a row	Intramuscular injection of 0.5 ml three times a day	Intramuscular injection of 0.5 ml, once a day, three days in a row	Intramuscular injection of 2.0 ml, once a day, three days in a row
4–90	Clinical observations and sampling				

The subcutaneous injections (Subcutaneous, SQ) of the drug were made in the fold of skin between the ears and neck (the loose skin behind the ear of male pigs), intramuscular — in the muscles of the neck, closer to the incision line. Disinfection of the working surfaces of the machine and the skin of animals was carried out by 'Clinosan' according to the manufacturer's instructions (Ukrainian-Polish Joint Venture 'ZVK' LLC, Lviv, UA). Carrier status in pedigree piglet males on ADV, PCV-2, and APP agents was determined by traditional standard methods (according to SOP NSC 'IECVM'). Shots, in a week and a half and within three months from the beginning of the trials, the veterinary service of farms took samples of stabilized blood and semen by standard methods in a week (5–9 days), two (14–20 days), and three months (52–64 days). The preputial smears were taken with the application of the necessary hygiene-technological procedures that limit their microbiological contamination (Althouse and Lu, 2005). The same requirements were observed in the sampling of nasal swabs and stabilized (by EDTA) blood. All collected samples were stored at fridge temperature and brought to the laboratory in the next 8–12 hours. At the end of the experiment, in the course of breeding work, stamping out one or two young boars from the most defective in each experimental group in terms of exterior indicators was carried out. The tissue samples of the lungs, spleen, and brain were taken for laboratory investigations. Swine pox was diagnosed by identifying the lesions. Lesions were round to oval, usually 1.5–2.0 cm in diameter (Fig. 1). The brown to black crusts were seen after the vesicle stage. Histological diagnosis was conducted by identifying typical, large, intracytoplasmic inclusion bodies in cutaneous lesions (Hess et al., 2011).

**Laboratory procedures.** The field biomaterials were primarily evaluated in group samples-analogs by the PCR method in the laboratory for molecular epizootology. To determine the number of positive pigs in the experimental group, additional immunodiagnostic studies in other laboratories according to SOP of the NSC 'IECVM' were carried out. In short, target viruses (ADV and PCV-2) were detected in blood leukocytes, nasal and preputial samples, lymph nodes, spleen, lung, and brain tissues by virus isolation technique in primary trypsinized cell cultures with isolate identification by passive haemagglutination test (PHA), home-tests with using of appropriate commercial diagnostic tools. To positive samples, 1–2 consecutive passages through ST cells subcultures were applied (for AD virus) or PAM (for PCV-2); to negative samples — 3 consecutive passages. For serologic analyses of blood samples on target-viruses correspondent commercial ELISA kits (IDEXX, USA) were used. Isolation of target bacteria (*A. pleuropneumonia*) was done by seeding samples on nutrient chocolate agar supplemented with 1% blood agar. The isolated bacteria, after Gram staining and

oxidative/fermentative (OF) test, were identified by determining the phenotypic characteristics by means of biochemical series of bacteriological differentiation discs (HiMedia, India).

Statistical data processing was done at the level of 95% (ANOVA) with the help of the software Statistica 7 (StatSoft Inc., 1984–2004). Animal experimentation was conducted within standard ethical norms.

Final conclusion about the carrier status of pigs was a result of the full-format analysis of virologic, bacteriologic, and serologic data on each experimental group. A seropositive carrier animal was considered recovered if three months after the last administration of the target drug, the titer of antibodies in her blood samples against the investigated agent decreased by at least 3 dilutions compared to the initial (before remediation).

**Results.** Test data of the cytotoxic activity of the CVD drug 'NanoViroSan-L/O' against subcultures of the primary trypsinized culture of ST cells, as well as against viruses AD, TD, PCV-2 and the causative agent of APP are summarized in Table 3. After standard treatment of native and infected test systems (cell culture or bacteriological medium) with an active composite in the dose range from 0.001 to 10.00 mg/ml (by Methisazone), it was established that the drug 'NanoViroSan-L/O' exhibits clear dose-dependent effect. The cytotoxicity of the composite was registered only at its concentrations of 5.0 mg/ml and above (according to Methisazone). It was manifested by signs of cell necrobiosis: massive pycnosis of their nuclei at concentrations of 5–10 mg/ml on 3<sup>rd</sup>–4<sup>th</sup> days of incubation of test cultures (ST cells and PAM) and massive rounding and exfoliation of cells on 4<sup>th</sup>–6<sup>th</sup> days of incubation. Moreover, these signs appeared faster, the higher the concentration of the composite was in the range of 5–20 mg/ml for Methisazone.

According to the results of these tests using the culture of ST cells, the intensity of the reproduction of the Aujeszky's and Teschen disease viruses sharply decreased already at the concentrations of the composite of 0.1 and 0.5 mg/ml, respectively, and in no case was manifested in the presence of the drug in the concentration range of 1.0–1.5 mg/ml according to Methisazone (Table 3). 'Teschovirus-like dynamics' was observed in the patterns of reproduction of the reference strain of circovirus in a culture of porcine lung macrophages. In this series of tests, no cytopathic changes were registered in the cell culture, but the intensity of accumulation of the circovirus antigen in it, according to the results of the passive hemagglutination reaction, sharply decreased in the composite concentration range of 0.1–0.5 mg/ml and in no case manifested itself in the presence of the drug in the concentration range of 1.0–1.5 mg/ml according to Methisazone.

The data of a series of studies using the reference strain of the APP pathogen, shown in Table 3, indicate

the dose-dependence *in vitro* the antibacterial action of the 'NanoViroSan' composite too. The 1<sup>st</sup> signs of such action were already registered in the range of drug concentrations of 0.1–0.5 mg/ml according to Methisazone. However, complete inhibition of this agent reproduction occurred at doses of the drug of 1.0 mg/ml and higher.

Table 4 summarizes the primary data from *in vivo* biotoxicity and antimicrobial activity laboratory testing of the drug 'NanoViroSan-L/O' in guinea pigs (n = 48). The obtained data *in vivo* indicate a similar dose-dependence of the drug action as it has *in vitro*. At the same time, antimicrobial activity against both AD and APP agents was already recorded at dose of 0.5 mg/cm<sup>3</sup> at the level of complete protection of all guinea pigs (n = 8) from these diseases — as well as at a dose of 1.5 mg/cm<sup>3</sup>. However, in guinea pigs, antibodies against the respective pathogens were detected on the 21<sup>st</sup> day after the last administration

of the drug at a dose of 0.5 mg/cm<sup>3</sup> (n = 2 from 4), but was not at 1.5 mg/cm<sup>3</sup> (no one from 4).

In clinical trials under production conditions, a consortium of the ADV and PCV-2 viruses with the APP agent served as target infectious agents. The clinical and epizootological manifestation of the associated infection of pigs with the participation of this consortium in the experimental pig farm was an acute respiratory syndrome in piglets of the growing group with feed conversion at the level of 3.8–4.1, lethality up to 30% and culling up to 15%, as well as infertility of sows up to 10% and sporadically cases convulsions among suckling piglets and their death with pronounced opisthotonus, with up to 83% of nests viability during 45 days from birth. In pigs of a distinct group, up to 20% of individuals registered minor clinical signs of the PMWS syndrome complex and up to 2% — PDNS syndrome complex.

Table 3 — Summarized primary data on cytotoxic and antimicrobial activity of the drug 'NanoViroSan' *in vitro* (n = 40, p ≤ 0.03), details in text

Targets	Time, hrs	Dose-depend effect of drug's target composite in final concentration in cultural mediums (by Methisazone), mg/cm <sup>3</sup>							
		0	0.01	0.1	0.5	1.0	1.5	5.0	10.0 <sup>***</sup> )
ST cells <sup>)</sup>	5	0000	0000	0000	0000	0000	0000	0000	0000
	24	0000	0000	0000	0000	0000	0000	0000	0000
	48	0000	0000	0000	0000	0000	0000	0000	0000
	72	0000	0000	0000	0000	0000	0000	0000	0000
	96	0000	0000	0000	0000	0000	0000	0000	0000
	120	0000	0000	0000	0000	0000	0000	00++	HHH+
	144	++++	++++	++++	++++	++++	++++	+H##	##HH
ADV <sup>)</sup> in ST cells	5	0000	0000	0000	0000	0000	0000	n.d.	n.d.
	24	++++	0000	0000	0000	0000	0000	n.d.	n.d.
	48	++++	++++	0000	0000	0000	0000	n.d.	n.d.
	72	####	####	++++	0000	0000	0000	n.d.	n.d.
	96	-	-	##00	0000	0000	0000	n.d.	n.d.
TDV <sup>)</sup> in ST cells	5	0000	0000	0000	0000	0000	0000	n.d.	n.d.
	24	++++	++++	0000	0000	0000	0000	n.d.	n.d.
	48	HHHH	++HH	00+H	H000	0000	0000	n.d.	n.d.
	72	####	####	00##	#000	0000	0000	n.d.	n.d.
	96	-	-	00--	H000	0000	0000	n.d.	n.d.
PCV-2 <sup>)</sup> in PAM	5	0%	0%	0%	0%	0%	0%	n.d.	n.d.
	24	25%	25%	25%	0%	0%	0%	n.d.	n.d.
	48	75%	75%	75%	0%	0%	0%	n.d.	n.d.
APP <sup>*)</sup> in bac. medium	24	25%	25%	10%	0%	0%	0%	n.d.	n.d.
	48	100%	100%	70%	25%	0%	0%	n.d.	n.d.
	72	100%	100%	100%	70%	0%	0%	n.d.	n.d.

Designations: <sup>)</sup> '0000' — absence of cytopathic action in any tubes; '++++' — destruction to 25% of monolayer in all 4 tubes; 'HH##' — destruction to 50% and more than 75% of monolayer in 2 tubes, respectively and presence of according agent's antigens; '0000' — presence of PCV-2 antigens without of cytopathic effect: 25–75%, volumes of cells from the total area of their monolayer that were positive for PCV-2 antigen in the immunoperoxidase test; <sup>\*)</sup> 25% — to 30 colonies; 70% — to 60 colonies; 100% — > 60 colonies; <sup>\*\*\*)</sup> rest checkered two concentrations (15 and 20 mg/ml) not show as were significantly more cytotoxic; n.d. — not did.



Table 4 — Summarized results of the ‘NanoViroSan’ toxicity evaluation and antimicrobial activity trial against ADV and APP agent in guinea pigs-model ( $p \leq 0.01$ ;  $n = 48$ )

Experimental groups (agents load)	Time, days	Disease signs appearance after ‘NanoViroSan’ injections in doses, mg/cm <sup>3</sup> :			
		0	0.25	0.50	1.50
1 — Guinea pigs without agents load, $n = 16$	1–90	‘0’ ( $n = 4$ )	‘0’ ( $n = 4$ )	‘0’ ( $n = 4$ )	‘0’ ( $n = 4$ )
2 — ADV (~ 3.0 lg TCID <sub>50</sub> on animal, intradermally), $n = 16$	1	‘0’ ( $n = 4$ )	‘0’ ( $n = 4$ )	‘0’ ( $n = 4$ )	‘0’ ( $n = 4$ )
	7	‘0’ ( $n = 1$ ), ‘+’ ( $n = 3$ )	‘0’ ( $n = 1$ ), ‘+’ ( $n = 3$ )	‘0’ ( $n = 4$ )	‘0’ ( $n = 4$ )
	14	‘+’ ( $n = 1$ ), ‘+++’ ( $n = 3$ )	‘+’ ( $n = 4$ )	‘0’ ( $n = 4$ )	‘0’ ( $n = 4$ )
	21	‘+++’ ( $n = 1$ ), ‘#’ ( $n = 3$ )	‘+’ ( $n = 1$ ), ‘+++’ ( $n = 3$ )	‘0’ ( $n = 4$ )	‘0’ ( $n = 4$ )
3 — APP (~ 5.0 lg BC on animal, intraperitoneally), $n = 16$	1	‘0’ ( $n = 4$ )	‘0’ ( $n = 4$ )	‘0’ ( $n = 4$ )	‘0’ ( $n = 4$ )
	7	‘#’ ( $n = 4$ )	‘0’ ( $n = 4$ )	‘0’ ( $n = 4$ )	‘0’ ( $n = 4$ )
	14	-	‘0’ ( $n = 1$ ), ‘+’ ( $n = 3$ )	‘0’ ( $n = 4$ )	‘0’ ( $n = 4$ )
	21	-	‘+++’ ( $n = 2$ ), ‘#’ ( $n = 2$ )	‘0’ ( $n = 4$ )	‘0’ ( $n = 4$ )

Designations: ‘0’ — absence of any morbidity reaction; ‘+’ — induration at the drug injection site for ADV and depression for APP; ‘+++’ — itching and necrosis of the skin at the injection site for ADV and bloody nasal discharge and exhaustion for APP; ‘#’ — death with typical disease pathomorphology.

So, pedigree male piglets, affected under the conditions of commercial pig farming by the above-mentioned associative microflora, were treated with the target composite drug according to five experimental schemes, which is aimed at optimizing the use of ‘NanoViroSan-L/O’ according to the method of administration and dose (Table 2). The obtained data are summarized in Table 5.

Table 5— Trial of ‘NanoViroSan’ activity on drift of male pigs,  $n = 43$ ;  $p \leq 0.023$  (by summary tests results — see text)

Time, days	Positive carrier status of experimental pedigree male piglets on Pseudorabies virus / Porcine Circovirus Genotype 2 / <i>A. pleuropneumonia</i> , animal number in subgroups					
	1 ( $n = 5$ )	2 ( $n = 5$ )	3 ( $n = 7$ )	4 ( $n = 5$ )	5 ( $n = 5$ )	Mock ( $n = 16$ )
0	3/3/4	4/2/2	5/3/3	2/4/5	5/3/3	9/12/14
≥ 7	0/3/4	2/0/2	0/0/0	0/0/0	0/0/0	-
≥ 14	0/0/-	0/0/-	0/1/-	0/1/-	0/0/-	-
≥ 21	2/1/1	1/1/0	2/1/0	0/0/0	0/0/0	11/14/9
≥ 35	2/2/0	-	-	0/0/0	0/0/0	-
≥ 90	2/2/3	2/1/0	1/2/0	0/0/0	0/0/0	14/14/7

Description: Groups No. 1–5: piglets 45-day-old age accordingly to Table 2; Mock: pedigree male piglets with the same status but without ‘NanoViroSan’ covering (was applicated a Amoxicillin protocol); ‘0/0/0’ — absence all of target agents; ‘2/0/0’ — presence of PCV-2 in two pedigree male piglets and absence all other target agents in all piglets of this experimental group; ‘-’ — test not done.

During the 3-month research, no signs of adverse effects of the drug ‘NanoViroSan L/O’ on pigs, under the conditions of traditional Ukrainian pig farming, were

registered. There no clinical signs of AD, PCV-2, and APP were registered during the entire period of observation among experimental animals aged 4.5 months, treated intramuscularly three times, once a day, for three consecutive days in a row in doses of 0.5 (group No. 4) and 1.5 cm<sup>3</sup>/20 kg (group No. 5). In the piglets of the remaining experimental groups treated intramuscularly three times in a dose of 0.5 cm<sup>3</sup>/20 kg with a minimum interval (3–4 hours, group No. 3), virus-carrying of AD and PCV-2 agents did not stop until the end of the 3-month experiment. At the same time, the carrying of the APP agent in piglets of group No. 3 was treated already on the seventh day after one-day three-time intramuscular treatment with an interval of 3–4 hours and was not restored until the end of the experiment. At the same time, viral carriage of AD and PCV-2 agents in this group stopped in a 2-week period after sanitation and then resumed In the groups of subcutaneous injection of the drug, the carriage of the pathogen of APP stopped only in piglets covered with drug three times with the highest interval, i.e. of 14–18 hours (group No. 2), and the virus carriage of the AD and PCV-2 in this group almost did not stop. The final results of the trials of three times subcutaneous administration with minimal intervals (group No. 1) were the same as in the control group of piglets (see Mock in Table 5).

In order to confirm the obtained results of laboratory and clinical studies, given above, at the initial and final stages of the studies, a retrospective analysis of the data of serological studies and herd zootechnical scoring was carried out. According to the results of this analysis, three months after the last administration of the drug, antibodies against the causative agents of AD and APP were detected in piglets groups of No. 2–5, but their antibodies against the causative agent of circovirus

infection were not detected in groups of piglets No. 4 and No. 5. Titers of antibodies against PCV-2 in groups No. 2 and No. 3 (dose of 0.5 ml, but different schemes of its use) during this period fell, on average, from 1:64.2 (n = 5, SD = 52.3%, p < 0.05) and 1:56.3 (n = 5, SD = 81.2%, p < 0.05) to 1:14.4 (n = 5, SD = 31.7%, p < 0.03) and 1:16 (n = 5), SD = 28.1%, p < 0.02), respectively. At the same time, during the test period (more than 3 months), the piglets of the 3<sup>rd</sup>, 4<sup>th</sup>, and 5<sup>th</sup> groups in terms of average live weight during control weighing exceeded the mock-piglets (n = 16) by 337 ± 57 g (n = 7), 1,220 ± 89 g (n = 5) and 1,170 ± 44 g (n = 5), respectively. In pigs of group No. 1 (a dose of 0.5 ml for a one-day subcutaneous injection three times a day), titers of antibodies against PCV-2 during the 3-month trials practically did not change: 1:67.6 (n = 5, SD = 85.2%, p < 0.05) versus 1:77.5 (n = 5, SD = 73.8%, p < 0.05), respectively. There was no difference in the

average weight of the animals of this group (n = 5) and mock piglets (n = 16).

Fig. 1 shows the results of testing the ‘NanoViroSan’ composite in a commercial pig farm for the eradication of swine pox in young animals. On the eve of the mating campaign and at the beginning of its implementation, one of four breeding boars and 5 of 32 sows from the nucleus of the herd were intramuscularly covered twice at the rate of 1 ml of the drug per 20 kg of body weight with an interval of 12–14 hours. In the course of the mating campaign and the entire next gestation period, no deviations in the state of health from the physiological norm were registered in the ‘NanoViroSan’ covered animals. After the birth of piglets (n = 38 in the experimental group and n = 189 in the control group, s. born from non-inoculated sows and boars), morbidity (by indicators of viability and skin rashes) was carried out by the staff of the pig farm.

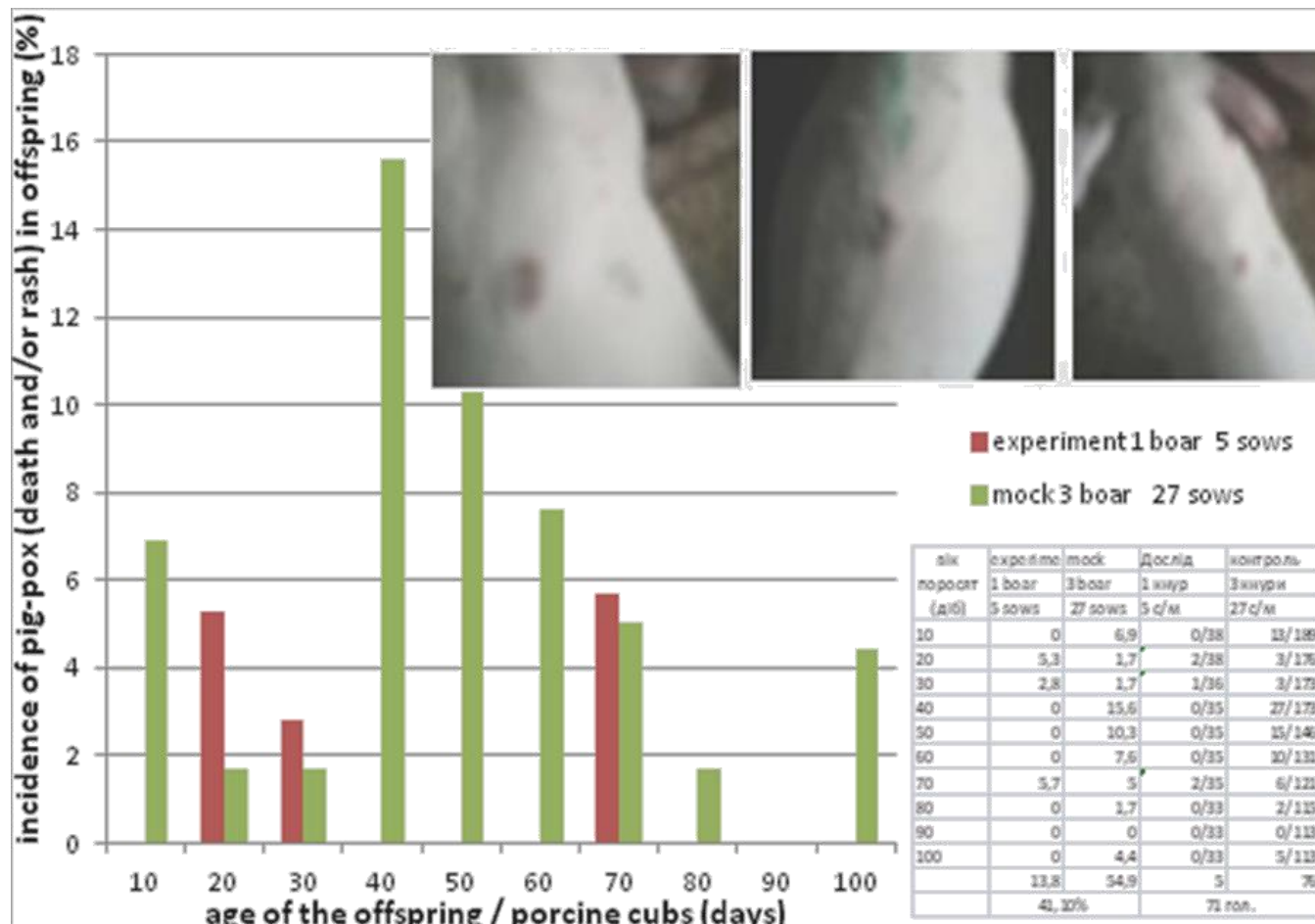


Figure 1. The results of treatment with the drug ‘NanoViroSan’ of the pig herd nucleus to prevent swine pox in the offspring (see the text for more details).

Unfortunately, this period coincided with the events of martial law, which were not compatible with conducting other, more evidential studies. However, the obtained data confirmed the significant effectiveness of the drug ‘NanoViroSan’ for the prevention of swine pox

in commodity farms (n = 227, p < 0.05). A feature of the therapeutic effect of the drug on piglets with pox was an increase in the size of individual papules as a result of its edema (cold painless edema, without loss of appetite and positive dynamics of feed conversion) in the first

5–7 days after the last (third) administration at a dose of 0.5 cm<sup>3</sup>/20 kg. Within 1.5–2.5 weeks, the animals fully recovered without additional interventions (n = 12), and when applied externally with zinc ointment — after 3–5 days (n = 8).

Based on the analysis of the obtained results and the experience of using ‘Lipozone’ (a drug-analog), the composite drug ‘NanoViroSan-L/O’ can be recognized as a universal drug and recommended for further trials in the current order for viral, bacterial, and mixed infections of pigs in dose 1.5–2.0 ml to adult pigs of 20 kg body weight intramuscularly three times with an interval of up to 24 hours, if necessary — repeat the scheme after a week or a half; for young pigs from 15 days of age — the same, but in a dose of 1.0 ml per 10 kg of weight.

Discussions and conclusions. An urgent problem of domestic pig farming is its high dependence, due to a number of diverse reasons, on the intensive use of a wide range of antibiotics (Linciano et al., 2019). For the past 40 years,  $\beta$ -lactam antibiotics have been the most sought-after veterinary support for animal husbandry — due to their wide spectrum of action, market availability and ease of use. However, their irresponsible use in poultry farming, animal husbandry, fish farming, etc., has caused global problems for public health — due to the emergence of resistance genes (ARGs) in pathogenic human bacteria.

Therefore, the priority task in this direction is the search and development of potentially clinically effective drugs alternative to these antibiotics, as priority (Linciano et al., 2019). Therefore, in the development of the target drug's antibacterial constituent, special attention was paid to search for inhibitors of microbial enzymes, the activity of which is related to zinc-containing coenzymes. These enzymes are deprived of a wide range of species of pathogenic bacteria and fungi to survive in adverse conditions, in particular in the presence of antibiotics. Such enzymes include  $\beta$ -lactamases of pathogenic staphylococci, *Klebsiella*, and enterobacteria. Fig. 2 (by Kim et al., 2013 — ‘bell-shaped’ structure in the center of the enzyme molecule) shows an example of the zinc-containing coenzyme of the most dangerous bacterial  $\beta$ -lactamase of the type ‘New Delhi’ NDM-1 (Kim et al., 2013). Other microbial enzymes possess similar zinc-containing coenzymes - for example, carbonic anhydrases (CA, EC 4.2.1.1) of gonorrhoea pathogens *Neisseria gonorrhoeae*, cholera *Vibrio cholerae*, ‘American trypanosomiasis’ (Chagas’ disease) *Trypanosoma cruzi*, pathogenic fungi *Cryptococcus neoformans*, *Candida glabrata* and *Malassezia globosa* (Supuran, 2021).

According to numerous data from the world literature, active inhibitors of these microbial enzymes are compounds of the chemical class of isatin-b-thiosemicarbazones (IBT). The thiol group of these

compounds (see in circle of Fig. 3) forms various coordination bonds with the zinc ions of the active site of the zinc-containing coenzymes as above — i.e. neutralizing of their (Li et al., 2021).

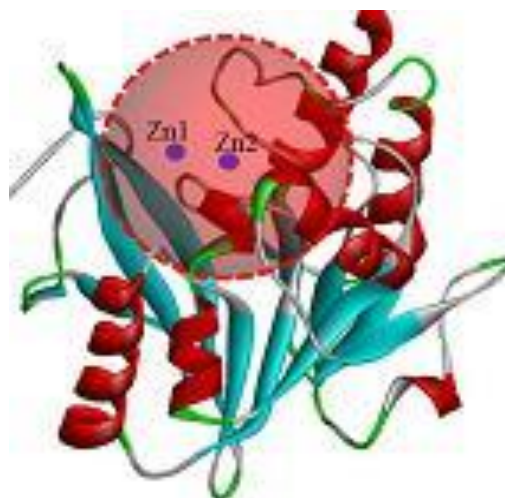


Figure 2. Structural model of the bacterial enzyme that ‘kills’  $\beta$ -lactam antibiotics (details in text).

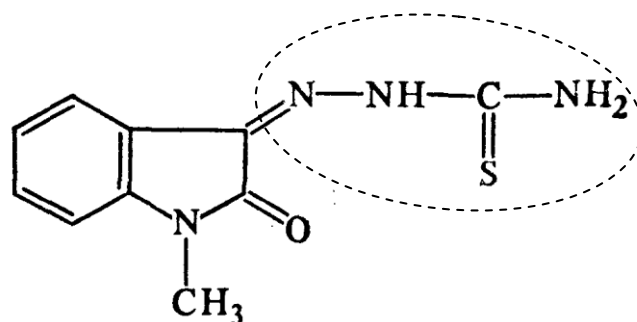


Figure 3. Structural formula of Methisazone.

For example, the highest level of inhibition effect of  $\beta$ -lactamases of methicillin-resistant *Staphylococcus aureus* (MRSA) by mentioned IBT-compound was showed in its inhibitory concentration of  $IC_{50} \leq 10 \mu M$  (Zhang et al., 2015). The specified concentration, according to the international database on the toxicity of chemical compounds, is 1,500 times lower than the index of toxic action of Methisazone, the active substance of which belongs to IBT class of chemical compounds namely (BV-BRC, 2022). Methisazone has been known since the 1950s as a virostatic agent with high therapeutic and prophylactic efficacy against smallpox in humans and currently, it is considered a domestic antiviral drug with a certain antibacterial effect (Lozjuk et al., 1996). It is well known that the antiviral activity of this drug is caused by blocking the expression of early proteins (Patskovsky et al., 1996) of many species of both DNA- and RNA-containing viruses (Levinson, 1975; Andreani et al., 1977, Bauer et al., 1970). Therefore, we chose Methisazone as a promising drug for the creation of a



composite drug with targeted action. To increase the activity of the target composite against bacteria and expand the antiviral spectrum, we applied the additional capabilities of Silgeran, the new drug of domestic origin.

The circumstances of the martial law in Ukraine did not allow us to conduct more detailed studies of the effectiveness of the drug — in particular, in a comparative aspect with analogous drugs of domestic and foreign production (i.e. conduct trial on therapeutically bioequivalence). However, the composition of the composite drug ‘NanoViroSan’ contains the medicals that have been obligatorily studied by its manufacturers — both at the preclinical and clinical levels during its state registration. That is, if the main task of the current tests is to study the compatibility of the components and the preservation of their therapeutic properties in the composite under production conditions, then the results obtained at a sufficient level of probability show a positive decision, provided that certain requirements are met regarding the method of use of the drug.

The obtained results of preclinical and clinical tests testify to the pronounced activity of the drug designed by us against the causative agents of Aujeszky's and Teschen diseases, type 2 circovirus, pig-pox as well as the causative agent of swine actinobacillary pneumonia. Its three-time intramuscular application made it possible to recover the pigs from carrying the specified pathogens. It is known that breeding boars are more resistant than other production groups to the clinical manifestation of infectious diseases — even to ASF (Bisimwa et al., 2021) and therefore, as a rule, serve as a hidden source of infectious agents. Moreover, from our unpublished data,

the 8 sows that we treated orally with this drug until stamping out (about 9 days) were healthy in the presence of all other untreated pigs in the ASF outbreak No. 428/2018 (SSUFSCP, 2022).

Therefore, the obtained results indicate the promising news of the injectable composite drug ‘NanoViroSan’ as a possible multipurpose agent in pig breeding. This meets the requirements of the modern world trend of medicinal chemistry for the development of multi-target ligands (MTDL), which is gradually changing the traditional methodological approach of ‘one drug — one target’ (Hashmi et al, 2021). Due to the harmonious combination of active substances in the drug, mainly antiviral (Methisazone, nanoxide of zinc) and antibacterial (Silgeran) direction, this complex remedy can be useful for use in emergency epizootic situations. For the bio protection of pig farming under the conditions of martial law in Ukraine, it is expedient to test the drug in enzootic focuses of ASF, in particular in small-scale and homestead farms that are located in zones of ASF spreading among wild boar population. According to our hypothesis, low virulent variants of the ASFV causative agent circulate in these zones as part of viral-bacterial associations (Buzun and Kolchyk, 2022). Summarizing, it is possible to predict with high probability that the complex use of composite preparations with probiotics, medical serums, and vaccines (primarily inactivated, from local strains of pathogens) will allow pig farming to quickly get rid of the current level of antibiotic load. In turn, this will significantly increase the agricultural export potential of Ukraine, as an influential participant in the fight against global hunger.

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