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EXPERIMENTAL EPIZOTOLOGY OF LOW-VIRULENT VARIANTS OF AFRICAN SWINE FEVER VIRUS

*African swine fever (ASF) remains an urgent problem of pig farming in Ukraine, the solution of which is possible only on the basis of deep scientific knowledge about the specific driving forces of the epizootic in its specific nozoareal. This is necessary in order to target anti-epizootic measures on the most vulnerable link of the epizootic chain in a specific nozoareal. The **aim** of the work was to develop a low-budget methodological base for experimental epizootology of low-virulent ASFV variants in Ukraine, in particular, to study the mechanisms of the formation of enzootic areas, quality control of anti-epizootic measures, and evaluation of the effectiveness of the antiviral drugs against them in Ukraine. **Methods.** Experimental and epizootological studies in the adaptation of suckling piglets to keeping in the* biosecurity-level *BSL-3 for laboratory animals (Patent UA No. 133248 dated 03/25/2019) were conducted at the laboratory base of the Odesa branch of NSC «IECVM». All procedures with infectious active biological materials in the current order were carried out in the BSL-3 module, built and certified with the assistance of the US Government in UAPRI (Odesa). The ASF agent strain «IECVM/Ternopil/2017» (infectious activity 4.0—7.5 lg* $HAdU_{50/cm}{}^3$*) circulating in the Ternopil region in 2017—2020 was used as a test virus. The presence of low-virulence variants of the ASFV pathogen in the studied samples was determined by a bioassay on suckling piglets, followed by three consecutive passages on a stable Vero line of the baby green monkey kidney cells. The isolated ASF virus was identified according to the methods and reagents recommended by the OIE Manual. **Results.** Intermittent passages «by the founder's method» of dilutions* 10^{-1} *and* 10^{-2} *of the ASF virus strain «IECVM/Ternopil/2017» on piglets (n=20) and the culture of porcine alveolar macrophages («*$ASFV^{PAM}$*») allowed us to identify highly-, moderately-, and low-virulent variants/clones in its composition. Verification by bioassay on suckling piglets (n=5) of low-virulent clones of the agent, which were stabilized in Vero cell culture («*$ASFV^{Vero}$*»), showed that after intraperitoneal infection at a dose of 4.25 lg* $HAdU_{50/cm}{}^3$*, they are capable of causing only a non-lethal*

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(within 2 weeks) viral infection with a maximum daily rectal temperature of 39.4±0.22 °C and duration of fever on average 1.6±0.14 days (5 of 5 piglets). Clones with greater virulence («$ASFV^{PAM}$») under similar conditions were able to cause a lethal infection with a maximum temperature of 40.7±0.37°C and duration of fever on average 3.9±0.27 days (17 of 20 piglets). Low-virulent clones were revealed by direct immunofluorescence in pulmonary and spleen smears of clinically healthy piglets on days 14 and 17 post-infection (p.i.); their antigens were visualized in Vero cells by indirect immunoperoxidase method after 48 h p.i. at dose about 0.01 lg $HAdU_{50/cm^3}$. They caused «crumbly» hemadsorption of infected Vero cells and their virions had typical for Asfarvirus view and size (210—220 nm). The obtained data served as the basis for analysis of the mechanism of rooting ASF agents in West Podillia enzootic foci, as well as for implication of the concept of low-budget quality control of anti-epizootic measures and evaluation of antiviral drugs' activities against ASF. ***Conclusions.*** *Low-cost operational procedures have been developed that allow one to use a vivarium of laboratory animals for ASF bioassay and meet principal requirements for science-based research in important aspects of experimental ASF epizootology. With their help, confirmation of the heterogeneity of the population of the ASF virus circulating in endemic foci of the Ukrainian Western Podillia was obtained ($p<0.05$, $n=25$). The developed methodological approach is suitable for the study of fundamental issues of ASF epizootology, as well as for the quality control of anti-epizootic measures against ASF. In particular, it is advisable to use it to improve the biosecurity of agricultural export programs in Ukraine, a country that is disadvantaged by ASF.*

Keywords: *African swine fever virus, biodiversity, virulence, bioassay, suckling piglets, agroexport, biosecurity.*

African swine fever (ASF) is one of the most dangerous animal diseases due to its devastating effects on current pig farming. Since the first report on Montgomery's disease (synonymous with ASF) in 1921, enough information has been accumulated to understand the sources and ways of spreading ASF, but measures to control and prevent it are still insufficient [1, 2]. The causative virus of ASF (ASFV) belongs to the DNA-containing arboviruses (without analogs in vet virology) of the *Asfarviridae* family [3], which in Africa cause asymptomatic infection among warthogs *Phacochoerus aethiopicus* and acute infection (panzootia) in African pig farming. For the Eurasian wild boar, the ASF virus is considered lethal, and its emissions from the fauna today pose the greatest threat to EU pig farming [4]. In Ukraine, ASF was registered from 2012: in the period until 2014 — as a sporadic cross-border infection, until 2017 — as an acute panzootia in the conditions of Moscow aggression (by a strange coincidence, similar to Georgia in 2008—2009), and in 2018, its epidemiology began to acquire signs of enzootic regularity [5]. Analysis of the current epizootic situation shows that from 2015, in the Eurasian nozoareal, ASF outbreaks are increasingly preceded by a long period of asymptomatic spread of the pathogen in the herd [6—8]. According to the literature, with a high probability, this may be due to the reformation of the nosological profile of the pathogen in favor of its low-virulence variants, with their inherent mechanisms of persistentia and vector transmission [9, 10].

According to the data from the 1970s and 1980s on the epizootic ASF in Spain, Brazil, etc., this phenomenon is characteristic of the «attenuation» of its acute phase: the spread of the disease slowed down, and the pathogen has become firmly rooted in enzootic foci. At the same time, Spanish scientists registered the appearance of low-virulence isolates of the ASF agent from the very beginning of the epizootic disease, and their share in the population of the pathogen has gradually increased over time [6—9].

According to the results of long-term research on African isolates, the epizootology of weakly- and strongly-virulent variants of the ASF pathogen has critical differences in anti-epizootic measures [10, 11]. The prevalence of ASF in the Eurasian nozoareal has similar patterns, which, due to the lack of available vaccines, require the improvement of other preventive measures and tools [12—14]. Therefore, in the current period,

it is advisable to direct the scientific support for anti-epizootic measures to improve procedures for the detection and use of low-virulence variants of ASF both in the assessment of the quality of anti-epizootic measures and in trials of the effectiveness of antiviral drugs.

The **purpose** of the present research is to develop a low-budget methodological base for experimental epizootology of low-virulent ASFV variants in Ukraine, in particular, to study the mechanisms of the formation of enzootic areas, quality control of anti-epizootic measures, and evaluation of the effectiveness of antiviral drugs against them.

Materials and methods

1. *Virus and antigenic materials*. The ASF virus is a strain «IECVM/Ternopil/2017», with an infectious activity of 4.0—7.5 lg $HAdU_{50/cm^3}$ in cultures of pig alveolar macrophages of lung and leukocytes of blood [15]. Some clones of this strain are able to be reproduced without adaptation in the permanent green monkey cell culture [16].

Commercial semi-purified antigen/cytoplasmic soluble antigen of ASF virus was produced by the European Union Reference Laboratory for African swine fever (product code «ASFV-Ag») in Valdealomos (Spain) [17, 18].

2. *Cell cultures*: green monkey established kidney epithelial cells line Vero was used in the form of 2—4-day monolayer cultures grown in test tubes and culture plates, as well as in cultured plastic flakes in seed concentrations, respectively, 500—600 thousand and 350—400 thousand cells/cm^3 [19]. Primary cultures of alveolar macrophage cells (AMC) and porcine leukocytes were grown in test tubes and plastic culture flakes and plates according to the CISA-INIA SOP and the OIE Manual [20—22]. For the cultivation of cells of the Vero line, the nutrient medium Eagle's DMEM (product D5796, Sigma-Aldrich/Merck, USA) was used; its growth variants contained 7—10% fetal bovine serum (product F7524, the same manufacturer). For AMCs and pig leukocytes cultivation, 0.5% lactalbumin hydrolysate (product 58901C, Sigma-Aldrich/Merck, USA) Hanks' Balanced Salt solution (product H9394, manufacture the same) was used together with 10—12% mix (1:2) of fetal bovine serum (see above) and sterilized by microfiltration of homologous porcine serum with erythrocytes (final concentration 0.8—1.0%; change each 3—5 days).

3. *Virus-specific sera*. Commercial reference sera against ASF (positive and negative for the virus) obtained from the European Union Reference Laboratory for ASF (products codes «ASF-CP» and «ASF-CN», respectively, [23]) in Valdealomos (Spain). Reference sera against CSF and PCV-2 viruses (positive, weakly positive/limit, and negative for these viruses) were kindly provided by the Polish NIS «PIWet» (Pulawy).

4. *Virological methods*. All work with the infectious agent of ASF was carried out in the current order in the BSL-3 module, built and certified with the help of the US Government in the State Institution «I.I. Mechnikov Anti-Plaque Research Institute of the Ministry of Health of Ukraine» (UAPRI, Odesa) according to Order No. 697/15.08.2017 of the State Food and Consumer Services of Ukraine and Permit No. 05.4-04/19/857-17/17462/26.06.2017 of the Ministry of Health of Ukraine, according to the requirements of the Standard Operating Procedure (SOP) of UAPRI No. 35/15.01.2018. This SOP was developed with our participation on the basis of methodological approaches laid down in the relevant SOP of the Reference Center of EU on ASF (Spanish NSC CISA-INIA, t. Valdealomos), as well as OIE recommendations [20—22]. The development of the regulations for keeping experimental pigs and working out certain elements of the procedures for obtaining macrophage cultures and conducting bioassays were carried out on the basis of the Odesa research station NSC «IECVM» and the Laboratory for studying pig diseases NSC «IECVM». All employees were involved in working with the infec-

tious agent of ASF only after mastering the rules of DSP 9.9.5.035-99 [24] and other guidance documents on biosafety in the current order,i.e., after certifying by the Regime Commission of UAPRI and clearance to work in the BSL-3 module by its administration.

Our approaches toASF virus isolation as a whole are protected by a patent of Ukraine [16] targeting for maximal save of the agent's population biodiversity and described in detail in the Institute's collection of scientific papers [25].

For retrospective analysis, it is very important to emphasize that a spleen sample from a slaughtered sow was taken by State veterinary service in the area where then other numerous ASF outbreaks were registered within 2017—2020. This is necessary to estimate the significance of natural heterogeneity/biodiversity of the isolate`s population for the ASF enzootic process evolution [26—28].

All samples were treated with antibiotics complex including Ceftriaxone Kabi (Labesfal Laboratorios Almiro, S.A., Portugal), 5 mg/mL + Gentamicin («Hemofarm» AD, Serbia), 7 mg/mL + Nistatin (PJSC «Borshchagovsky plant»), 5 mg/mL, solvent Eagle medium (DMEM, HiMedia™ Laboratories Pvt Ltd, India), pH 7.2—7.4; exposure 40—60 min., clarification by slow-speed centrifugation followed by microfiltration through a syringe filter with a pore diameter of 0.2μm (Millex®, sterile, 4/13/25 mm; Merck KGaA, Germany). Virus titer was determined on sensitive cell cultures by the standard Kerber titration method (1931) in hemadsorption units ($HAdU_{50/cm^3}$). The number of pig erythrocytes adsorbed on ASF-infected cells through adsorption of 10 or fewer erythrocytes per infected cell (accounting with use of a Zeiss Universal microscope) was considered «crumbly» [29, 30].

To isolate low-virulence variants of the ASF agent, the method of analyzing the heterogeneity of the population of the isolated live particles of the ASF agent according to the principle of «genetic founder» was used [31]. The criteria of VNIIVViM (of the former USSR) were used to assess the virulence of the virus strain and its clones according to clinical and pathological signs of ASFV, correlated with the dose of infection of animals according to the hemosorbing activity of the pathogen *in vitro* [29, 30]. We implemented these techniques in our modified bioassays on suckling piglets (n=9) up to 7 days of age that were treated with sedative tabs. They were infected with virus-blood of the strain «IECVM/Ternopil/2017» of the ASF pathogen (infectious activity in the culture of alveolar macrophages 4.0 lg $HAdU_{50/cm^3}$), taken at a dilution of 10^{-2} on PBS at a dose of 1.5 cm^3 (Fig. 2). A highly virulent clone of the strain «IECVM/Ternopil/2017» was considered its isolate from suckling piglets that died with signs of opisthotonus (fulminate form of ASF) in the first 36 h post infection (p.i.). A moderately virulent clone of the strain «IECVM/Ternopil/2017» was considered its isolate from piglets that died with signs of fever 40.5 °C and above for 2 or more days, accompanied by other manifestations of acute ASF (hemorrhagic diathesis, cyanosis of the skin of the torso, hooves and/or ears, etc.) without lethality in the first 7 days p.i. A weakly virulent clone of the strain «IECVM/Ternopil/2017» was considered its isolate from suckling piglets that did not die for 2 weeks p.i. and were clinically healthy at the time of diagnostic slaughter within the 14th—17th days (observation period), although they showed some clinical signs in the first phase of the disease (periodic fevers up to 40.5 °C, bouts of depression, digestive and/or respiratory disorders, etc.).

5. *Biosafety control of laboratory work on ASF.* Outside the UAPRI, the hemadsorption test (HAd-test) was used to control the absence of hemadsorbing infectious agents on batches of all cell cultures involved in trials before and after infection with field samples.

6. *Identification of viruses, viral antigens, and antibodies against them.*

6.1. Viroscopy of cellular detritus was performed by the classical method of transmission electron microscopy (TEM) with a subject magnification of 20.000 × and 40.000 ×. For reasons of biosafety of cellular detritus after the adsorption procedure on EM-meshes fixed by 2.5% solution of glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2), followed by post-fixation with 1.0% aqueous solution of OsO_4; staining was performed with uranyl acetate [32].

6.2. Immunoperoxidase test (IP-test) used for the identification of ASF virus antigens in the transplanted Vero-cell culture according to current SOP of NSC «IECVM» Research laboratory for pig diseases for the isolation and identification of swine viruses. Appropriate components of commercially available commercial ELISA diagnostic kits were used in the IPM as diagnostic sera and immunoperoxidase conjugate. These reagents preliminary were titrated on the reference test slides and then used in the dilutions. They were mainly used to treat cell culture grown on Linbro culture plates and fixed with 96% ethanol mainly within the 20th—60th h p.i. The fixed and washed culture plates were dried and stored for further staining at a temperature of minus 8—12 °C for up to 1 month.

Incubation with a working dilution of specific and normal sera (mainly 1:50—1:80) was performed at a temperature of 37.0 °C, followed by 3-time (5 min) washing with buffered saline (PBS) with pH 7.2—7.4. Then cell culture preparations were similarly incubated in plates with commercial immunoperoxidase conjugate against porcine IgG taken at a working dilution (mainly 1:150—1:300). A mixture of hydrogen peroxide (final concentration 0.01%) with o-dianisidine (final concentration 0.5 mg/mL) prepared ex tempore was used for the development of peroxidase reaction.

6.3. Solid-phase enzyme-linked immunosorbent assay (ELISA) was performed according to the manufacturers of leaflets-tabs of ELISA kits on the standard ELISA equipment (thermostat, mixer, reader, etc.). For ASF trials, a registered commercial kit for detection of antibodies against ASF (ELISA TF based on recombinant p30 antigen «SVANOVIR® ASFV-Ab Asser Svanova», Sweden) was used. To monitor concurrent infections and exclude non-specific colorations, commercial kits for the detection of antibodies against CSF, PCV-2, PRRS, and Mannheimia haemolytica registered in UA were used. As well, porcine serums antibodies to the mentioned agents were detected by commercial kits «IDEXX CSFV Ab Test»(IDEXX, USA), «PCV-2 ELISA SK 105» (BioChek BV, Netherlands), and «IDEXX PRRS X3 Ab Test» (IDEXX, USA), respectively. Serum antibodies to somatic antigens of *Mannheimia haemolytica* were detected by the classical test of the bacterial agglutination inhibition using formalin-killed 22-h cultures of *P. haemolytica* serotype 1 as antigens [33]; as reference sera, »Serumpositive» and «Serumnegative» from ELISA kit, «Test ELISA pour le diagnostic sérologique de Mannheimia haemolytica» (Bio-X Diagnostics, Belgium) were used.

6.4. Immunofluorescence assay (IFA) was used to identify ASF antigens in impressions of samples (lung, spleen, spleen, kidney, and lymph node) from pigs. For this, the FITC diagnostics «Monoclonal antibodies 18 BG3-FITC on vp72 protein» (Ingenasa, Spain) was used. Impressions of sections of the studied organs were made in 3 replicates on degreased slides, dried, and fixed by cold acetone for 15 min or by 96 ethanol for 1 h at RT and then followed the leaflet-tab of the manufacturer.

After washing PBS (pH 7.2—7.4, 3 times for 5 min), the prints were incubated with FITC-Mab conjugate (dilution 1:250—1:600) at 37 °C in a humid chamber for 30—40 min and washed three times with PBS. The results were registered on an XS-8530 fluorescent microscope (Shanghai Sanshen Medical Instrument Co., LTD, China) in the ultraviolet light range (AFITC = 496 nm).

Experiments conducted on animals did not contradict the current legislation of Ukraine

(Article 26 of the Law of Ukraine 5456-VI of 16.10.2012 «On protection of animals from cruel treatment») and «General ethical principles of animal experiments» adopted by the First National Congress on Bioethics (Kyiv, 2001) and corresponded to the international bioethical standards (materials of the IV European Convention for the protection of vertebrate animals used for experimental and other purposes, Strasbourg, 1985) [34]. The research program was reviewed and approved by the Bioethics Commission of NSC «IECVM» in the current order.

Results. *Development of a standard procedure for keeping pigs in the vivarium of laboratory animals*. On the laboratory base of NSC «IECVM» and its branch of the Odesa Research Station, a method of adaptation of suckling piglets to keeping in the vivarium level BSL-3 for laboratory animals has been developed. Low-budget means of maintenance and manipulation were selected (Fig. 1, *a—d*). Also, a standard procedure including artificial feeding of suckling piglets from 3 to 20 days of age with «Phenazepam-IC» has been developed. «Phenazepam-IC» is a tranquilizer of the benzodiazepine derivative group («Interchem. ltd», Odesa, UA); target dosage 0.005—0.015 mg per 5 g of milk powder. As further research showed (see below), this, in summary, allowed developing optimal conditions for low-budget bioassay for ASF in compliance with all necessary biosafety requirements, as well as to create innovative approaches to studying ASF biodiversity, i.e., heterogeneity of its populations. The full cycle of the research (strain isolation and population analysis) was conducted on 25 suckling piglets (Fig. 2); in particular, 11 of them were used to isolate an enzootic virus with maximum preservation of its biodiversity (which is ensured by infection of only the natural biological host, i.e., the pig organism) and to study the virulence of the «IECVM/Ternopil/2017» strain, i.e, the general virulence of all its clones/virions' populations). This is important for the formation of a methodological base for forensic and veterinary investigations of ASF outbreaks and biosafety ensuring agrarian export-import operations in Ukraine.

The results of the use of suckling piglets for the isolation of high- and moderate-virulence variants of the ASF agent. In the first 38 h p.i., piglets inoculated with the first dilution of 33% spleen suspension (group «10^{-1}», n=3) showed a fulminant form of ASF (Fig. 2). Piglets inoculated with the second dilution of this primary material (group «10^{-2}», n=5) for the 3rd—5th days p.i. showed an acute form of the disease with the minimal hemorrhagic syndrome (n=4), and one piglet manifested chronic illness. The mortality of the illness in both groups was the same. Piglets (n=2) of the next, «second line», which were inoculated with a cultural clone (PAM) of the agent (dose 1.5×3.75 lg $HAdU_{50/cm}{}^3$), derived from piglets of group «10^{-1}» (first passage) in the first 38 h p.i., like their predecessor, showed the pattern of fulminate ASF. The piglets (n=3), which were inoculated by cultural material (PAM) of the agent from group »10^{-2}» piglets, i.e., from piglets with chronic illness (Fig. 2) in dose 4.0 lg $HAdU_{50/cm}{}^3$, developed acute ASF during the next 4th—7th days p.i.

Improvement of approach to isolation and identification of the low-virulence variants of the ASF agent was carried out on the basis of the data of VNIIVVIM (former USSR) and PIADC (USA) on the selective properties of green monkey cell cultures regarding similar variants/clones of Asfarvirus [29, 35]. For reconstitution of strain biodiversity of suckling piglets (n=9) infected with the cultural virus (PAM-variant, dose 1.5×3.75 lg $HAdU_{50/cm}{}^3$ intraperitoneally). Two of them died for one day p.i. with signs of the fulminate form of ASF. Three suckling piglets within 27—36 h p.i. demonstrated the signs of constant fever of 40.5—41.3 °C, cyanosis of the hooves (Fig. 1, *e*) and/or skin of the ears, chest, and/or tail. The other two piglets developed signs of damage to the respiratory system such as shortness of breath, wheezing, and in the ter-

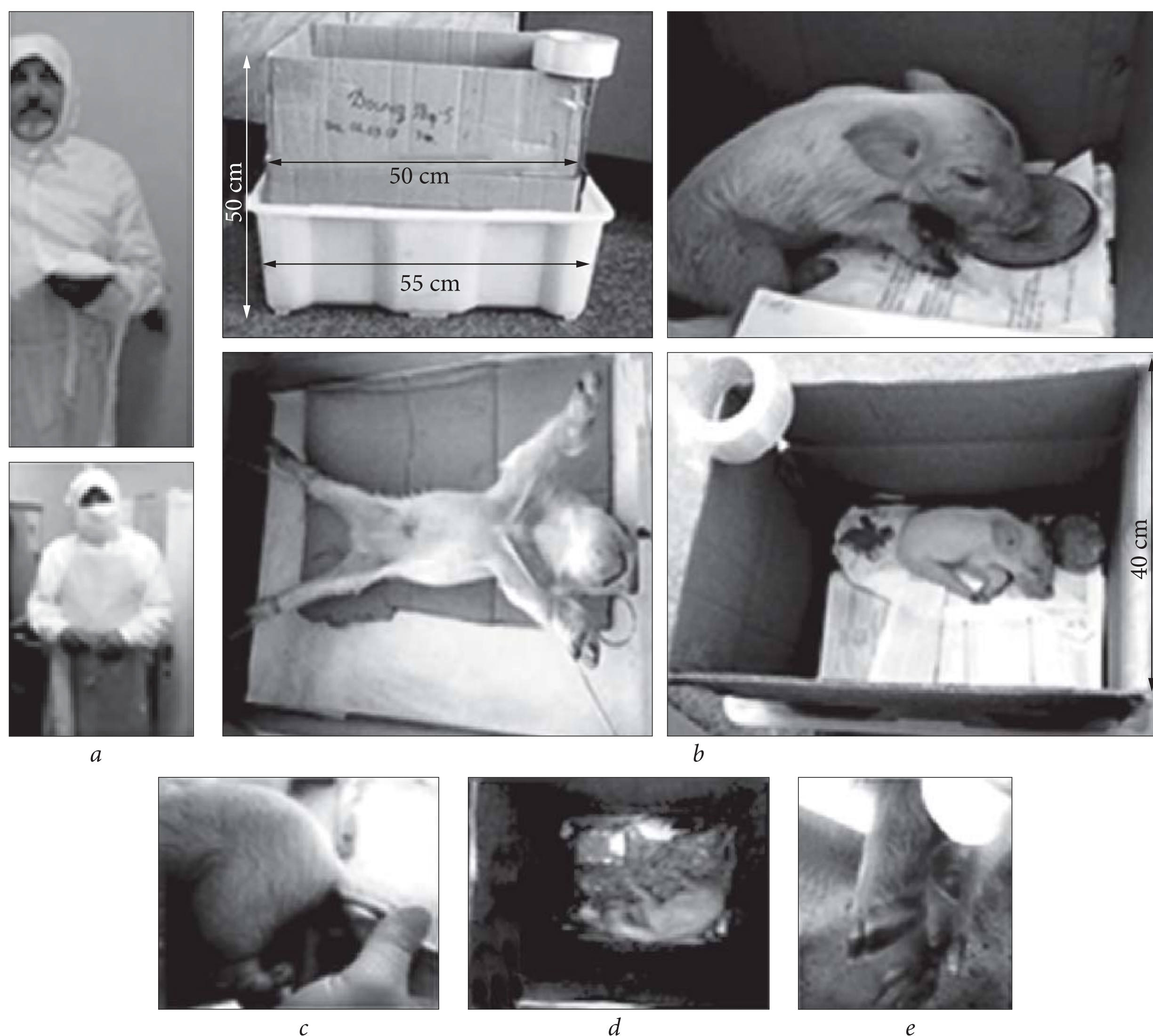

Fig. 1. Collage of pictures of the results of validation of the Standard operating procedures of isolation and evaluation of the activity of the low-virulent variant of the ASF pathogen: (a) the use of anti-plague uniform No. 3, 4 and (b) equipment for piglet holding on milk powder with «Phenazepam-IC» to work with the ASF virus in an infectious vivarium for laboratory animals of the BSL-3 level biosafety; (c) rectal thermometry of infected piglets; (d) suppression of infected piglets during recurrent fever on the 4th and 9th days p.i., and (e) cyanosis of the hooves on the 13th day p.i. by the low-virulent variant of strain «IECVM/Ternopil/2017»

minal phase — bloody discharge from the nose. In summary, all these 7 piglets died within the 4—8 days p.i. (acute pattern of ASF).

Two infected suckling piglets did not die within the 10th—12th days p.i. (observation period) and did not show any signs of illness. They were euthanized on the 12th and 14th days p.i., and the antibodies against the ASF virus were revealed in them by serological investigation. A cytopathic hemadsorbing agent was revealed in the bone marrow and spleen of piglets (both sample-analogs) at the first passage in Vero cell culture. It was identified as an ASF agent by the immunoperoxidase test (Fig. 3, *a*) and TEM. At

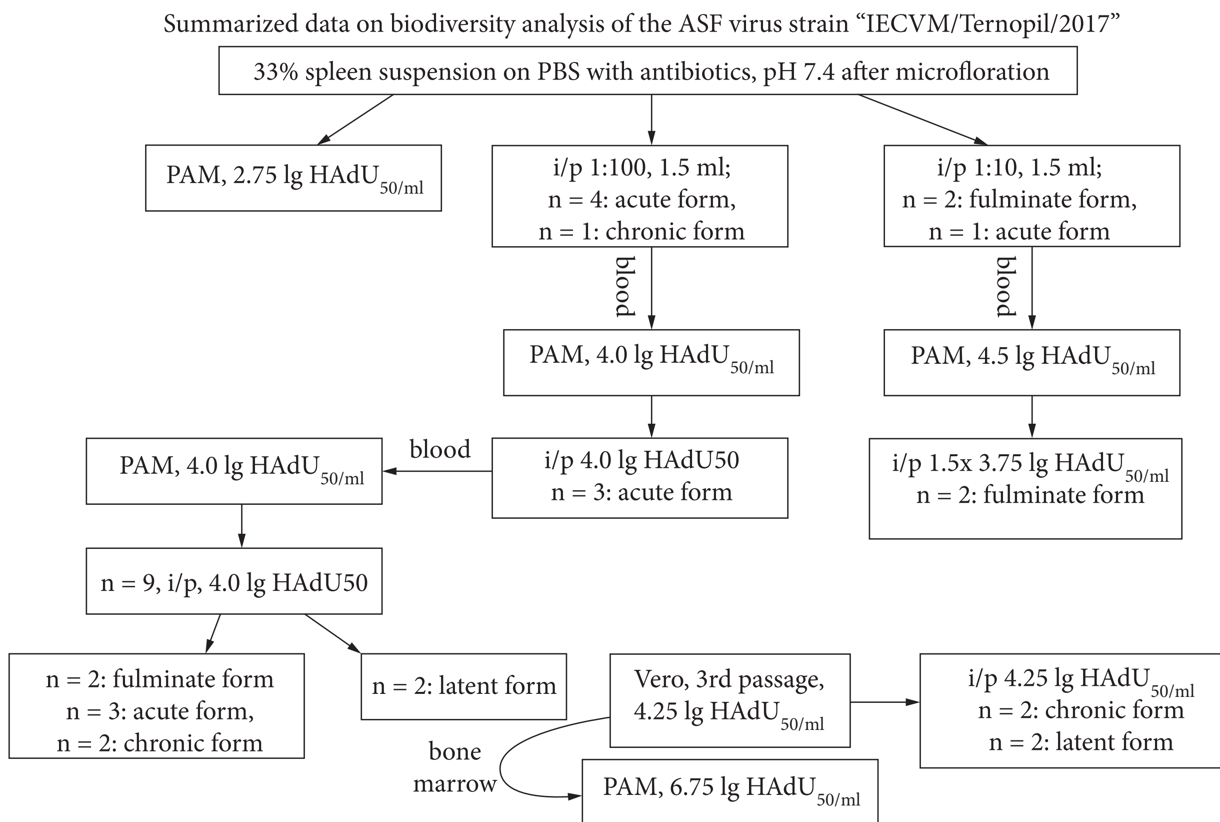

Fig. 2. Summarized results of population analysis of the strain «IECVM/Ternopil/2017». Dark boxes — the chain of procedures for isolation of high and moderate virulence clones of the strain; semi-dark boxes — the chain of procedures for isolation of the low virulence clones. Light arrows — infection of cell cultures with tissue samples from piglets. Dark arrows — infection of piglets with the cultural virus. Marks: PBS — phosphate-buffered saline; PAM — porcine alveolar macrophages culture; i/p — intraperitoneal injections of suckling piglets; $HAdU_{50/cm}{}^3$ — dose of i/p; $HAdU_{50/ml}$ — virus titer in hemadsorbtion units

the third passage, its titer in Vero cell culture reached 4.25 lg $HAdU_{50/cm}{}^3$ («$ASFV^{Vero}$» clones).

This agent caused the «crumbly» hemadsorption of swine erythrocytes by criteria of VNIIV-ViM (the former USSR) and Plum Island Center [30, 35]. Fig. 3a shows that at the 48th h p.i. (dose about 0.01 lg $HAdU_{50/cm}{}^3$), the monolayer of Vero cells still intact (transparent, as seen on uninfected cells between clusters of virus-infected/stained Vero cells), antigenic material of the virus after 24 h was detected by the immunoperoxidase method exclusively in cell nuclei (not shown), and after 72 h p.i., it was evenly distributed throughout the cell.

These five suckling piglets were infected with mentioned «$ASFV^{Vero}$» intraperitoneally, at a dose of 1.5 mL for proof of a selective effect on the low-virulent clones of ASFV passages in Vero cells. On the 14th (n = 2) and 17th (n = 2) days p.i., piglets remained clinically healthy. Only from 3 to 8 days p.i., two piglets had clinical signs of recurrent fever with peaks of 40.0—40.2 °C which was accompanied by depression, and one of them up to the 13th days p.i. had cyanosis of the hoof horn (like Fig.1, *e*). The three other piglets did not manifest any illness signs: they were euthanized on the 14th day p.i., and the antibodies against ASF virus were revealed by the serologi-

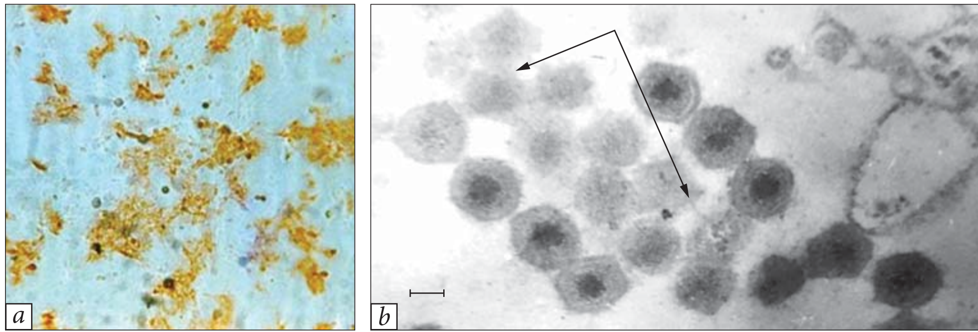

Fig. 3. Reproduction of low-virulence ASF virus clone in Vero cell culture, the third passage: (a) visualization of viral antigens in Vero cells by the indirect immunoperoxidase method, magnification about 650 ×; (b) cellular detritus viroscopy at magnification 11 × 40000, dash left = 100 nm: enclosed with arrows — a cluster of low- stained virions (see the text)

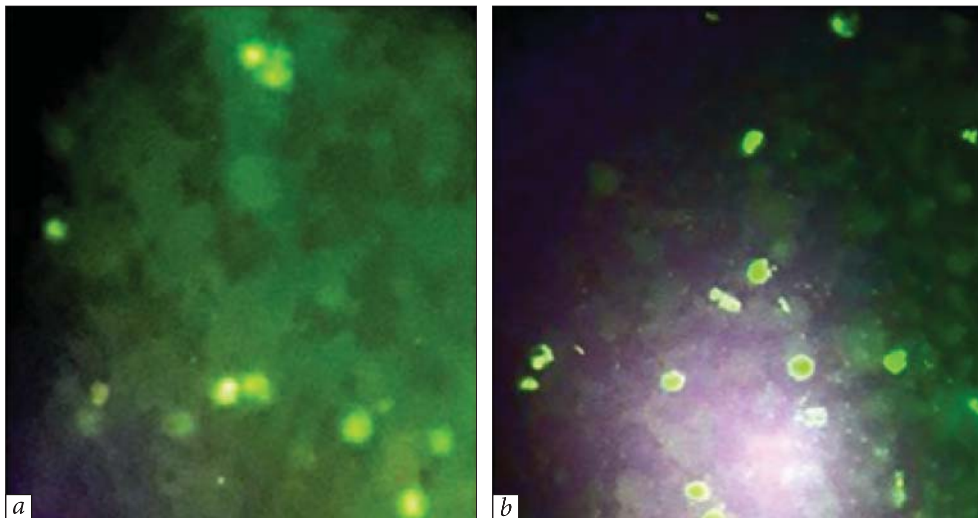

Fig. 4. (a) Pulmonary and (b) spleen smears of clinically healthy piglets on the 14th and 17th days p.i. with an ASFV clone; propagation in Vero cell culture («ASFV^{Vero}»): (a) visualization of viral antigens in alveolar macrophages and (b) splenetic lymphocytes by direct immunofluorescence assay

cal investigation. Also, a cytopathic and hemadsorbing agent was revealed in the bone marrow and spleen of piglets (both sample-analogs) at the first passage in Vero cell culture.

According to the results of IFA, alveolar macrophages (Fig. 4, *a*) and splenocytes (Fig. 4, *b*) of these clinically healthy piglets contained antigens of the ASF virus. IFA using antiviral FITC-diagnostics (direct method) in our investigation was most expressed (during staging, including the preparation of test slides) and did not exceed three hours. At the same time, the immunoperoxidase test was nonspecific due to the high level of background reaction of endogenic peroxidase in the porcine organs.

As seen, the bone marrow isolate of virus from three infected suckling piglets of this experimental cluster («latent/unapparent ASF infection») that was isolated in Vero cell demonstrates the same cultural properties as «ASFV^{Vero}» clones

used for these piglets inoculation. The viroscopy of cellular detritus of the first passage material of this isolate/clone (Fig. 3, *b*) was carried out exclusively for morphological identification of the virus. There was established that «ASFV^{Vero}» clones contain virions with typical morphology for Asfarviruses but with some special features (preliminary data). Their diameter ranged from about 210 to 220 nm while the diameter of the «parental» virus (strain «IECVM/Ternopil/2017» per se) ranged from about 180 to 300 nm. In both instances, poorly-stained virions were detected in addition to the contrast-stained virions. They may be defective virus particles, which requires additional study.

Table 1 summarizes the results of comparative analysis of the virulence of «ASFV^{PAM}» and «ASFV^{Vero}» clones of the strain «IECVM/Ternopil/2017» for suckling piglets according to the criteria for 80—90 kg pigs used in the VNIIVViM (former USSR [29, 30]) and Plum Island Animal Disease Center (USA [35]).

Table 1 shows that by the model of suckling piglets, the main clinical and experimental indicators in which low-virulent clones of the ASF agent differ from highly and moderately virulent ones are the time to death following 14 days p.i. (difference of 100%), fever duration (difference of about 75%), and Max daily temperature (difference of about 75%).

Since, according to Table 1, the virulence of «ASFV^{Vero}» clones met the criteria of low-virulent variants of the ASFV agent [35], it can be concluded with an acceptable level of probability (p≤0.05, n=25) that the passage of strain «IECVM/Ternopil/2017» in the Vero cells line allows for identifing clones with low virulence in its population.

Hence the results obtained permit us to conclude that the criteria for low virulence of ASF virus clones in the model of suckling piglets are the absence of their death within 14 days after intraperitoneally infection at a dose of about 1.5× 4.0 lg $HAdU_{50/cm^3}$, as well as seroconversion to the ASF agent with the presence of viral antigens in alveolar macrophages and/or spleen (pulmonary and spleen tissues).

Implementation of the obtained clinical and experimental data. The results of the above studies demonstrate a powerful possibility to study important items of ASF epidemiology and biotechnology by our methodological approach based on maximizing conservation and analyzing the biodiversity of ASF isolates.

First of all, based on the results of the above studies, the NSC «IECVM» Standard Operating Procedure (SOP) for quality control of disinfectants and drugs against ASF has been developed. According to it, the DZPT-2 disinfectant developed at the NSC «IECVM» has been validated [36], and the validation of the «NanoViroSan» line of antiviral drugs has begun, in particular, for preventive treatment of pigs in ASF protection zones [37].

Table 1. **Comparison of suckling piglet's survival after inoculation with «ASFV^{PAM}» and «ASFV^{Vero}» clones of the strain «IECVM/Ternopil/2017» following intraperitoneally inoculation***

Virus and dose	Number of survivors**/ total	Mean (SD)			
		Time to death (days)	Fever		
			Number of days to onset	Duration (days)	Max daily temperature (°C)
«ASFV^{PAM}» clones, 1.75—3.50 lg $HAdU_{50/cm^3}$	3/20	5.8 ± 0.68	1.7 ± 0.63	3.9 ± 0.27	40.7 ± 0.37
«ASFV^{Vero}» clones 4.25 lg$HAdU_{50/cm^3}$	5/5	0	1.2 ±0.39	1.6 ± 0.14	39.4 ± 0.22

Note: * — p≤0.05, n=25; ** — on the14th day p.i.

Proposals for the State Food and Consumer Service of Ukraine (project) to improve the quality control of disinfection in ASF outbreaks have been developed as well, in particular, to add for using the drug DZPT-2 to final disinfection in order to inoculate suckling piglets with specially prepared extracts of samples from critical control points (CCP) of the object of veterinary supervision (OVS). By our approach, inoculated animals are kept on the OVS territory as sentinels (instead of 70—80 kg pigs), and after 7—10 days p.i., they should be investigated for ASF and concurrent microflora by laboratory tests (serology and PCR). According to preliminary estimates, the use of these methods will provide a significant increase in the effectiveness of measures against ASF in Ukraine.

Another practical aspect of using the data obtained in this study is the epidemiological analysis. In our case, it is possible to retrospectively assess the influence of biodiversity of the «founding strain» on the dynamics of the epizootic process of ASF in the Ternopil region. The most valuable information is that the date of the disease in the pig from which the «IECVM/Ternopil/2017» strain was isolated corresponds to the first officially registered outbreak of ASF in the Ternopil region (No. 275/22.10.2017). In the period 2017—2020, ASF outbreak No. 275 was the first on the territory of Berezhany Opillia (Site-center location in decimal degrees: Longitude 49.446524 Latitude 24.854004[38]) and adjacent lands of Western Podillia with a total epidemic area of up to 5000 ha, where 13 more outbreaks of ASF were officially registered additionally [39]. This gives reason to consider the «IECVM/Ternopil/2017» strain as the «founding strain» of the specified enzootic focus of ASF. Outbreaks Nos. 285, 287, and 288 appeared in 2017; outbreaks Nos. 326, 327, 366, 373, 388, and 444 — in 2018; outbreaks Nos. 474 and 291 — in 2019, and Nos. 521 and 526 — in 2020. Moreover, four of them (Nos. 285, 287, 288, and 327) — in wild boar populations over 9 months of 2017—2018, that is, only in the initial phase of the ASF epizootic process in the Ternopil region. Since 2018, no ASF outbreaks have been registered in wild boar populations, while 10 ASF outbreaks have been registered in backyards and small pig farms within the Opillia and adjacent lands of Western Podillia.

A similar pattern of ASF is typical for Central Africa's nosoarea: there, in the enzootic centers, the source of the pathogen is wild pigs of various species and domestic pigs — carriers of low-virulent variants of the ASF virus [40, 41]. Thus, retrospectively, it can be assumed that outbreak No. 275 became an important prerequisite for the formation of a stationary enzootic center of ASF. Therefore, following the retrospective analysis, the results of studying the biodiversity of the «IECVM/Ternopil/2017» strain can be interpreted as a definite confirmation of the hypothesis about the rooting of the ASF agent, especially in wild pigs (at least in Opillia within 2017—2020) due to the accumulation of low-virulence variants in the ASF virus field population. This poses a great threat not only to Ukrainian but also to Polish pig breeding since the Meridian Opillia Ecocorridor is connected to the Latitudinal Galicia-Slobozhanskyi Ecocorridor, through which cross-border migration of wild boars, latent carriers of the ASF agent, takes place. This analytical data support the promise of using our approach for epidemiological studies.

Our approach would be even more productive if it were improved in the direction of quantitative assessment (in percent) of biodiversity of viral clones of populations of the ASF agent by virulence.

Discussion. Control of farm animal diseases is constantly complicated by the influence of changing environment on the circulation of pathogens of various etiologies [42, 43]. This is directly touching the problem of ASF eradication in the current period of development of its Eurasian nozoareal. This is evidenced in particular by scientific data on the plasticity of this

disease pathogen depending on the ecotype of enzootic areas [44, 45]. Antigenic diversity of the ASF pathogen is manifested in the formation of group-specific immunity against its variants of the corresponding serogroup: this is based on the suppression of group-specific antibodies' hemadsorption of the pathogen in cell cultures [46]. Different ASF genotypes with different virulence have been linked to different encores of sub-Saharan Africa and around the world, and understanding the mode of the genotype circulation is important for studying its phylogenetic relationships and improving prevention and control strategies [47—49]. In its Eurasian nozoareal, issues of associative activity of the causative agent of this disease with viruses and bacteria of certain species appear on the agenda. In this direction, there are certain solutions to the problems of the spread and rooting of low-virulence variants of the pathogen, in particular, within the framework of our Stop ASF mix concept [47]. The influence of competitive microflora on the adaptation of the ASF pathogen to the ecosystem (rooting in nozoareal) is absolutely indisputable, but today, it is practically completely ignored, at least in the accessed information. Regarding competing microflora, we saw that in no less than 3 of 7 cases of ASF Pasteurella hemolytic were present in samples from pig farms, in our opinion, in enzootic areas, where the State vets effectively detected the virus. According to our data, outbreaks of ASF in these farms occurred shortly after the implementation of measures against Pasteurellosis (to be published). This drove us to consider the possible participation of some bacteria in the selective influence on respective variants of the causative agent of ASF, in particular, through influence on the reproductive activity. In our opinion, an important step in this direction, is to find out whether there are such options for the field agent circulating in Ukraine.

The results described in the current publication indicate a clear biological heterogeneity by the pathogenicity of the studied ASFV population. Since this population dates back to the initial phase of the ASF epidemic in Western Podillia (2017), according to the Sorbonne University's mathematical model, it can be considered an epidemically unpredictable (stochastic) «*starting cluster*» in the initial period of the epidemic outbreaks [50]. However, there for the following three years, more than 14 officially registered outbreaks of ASF (among domestic and wild pigs) adjacent to the primary outbreak else to the territory of Opillia (area of Western Podillia) were added, with a total threatened area of up to 3.000 hectares. Therefore, according to the OIE Terrestrial Animal Health Code (2022), ASF should consider this area an enzootic area. Our analysis of the clones' population of the strain «IECVM/Ternopil/2017» (as mentioned, «starting cluster») showed that, in addition to highly pathogenic variants, it also contains low-virulence variants. Therefore, the obtained results indicate the need to include the factor «biodiversity of the agent population» in the specified epidemiological model in order to increase the predictability of the epidemic process of ASF. This is consistent with the current data on the epizootiology of ASFV, which increasingly support the argument about the gradual replacement of highly virulent variants of the ASFV pathogen in enzootic centers with low-virulence ones [51]. The obtained data also provide grounds for searching in the Opillia enzootic area for a natural avirulent variant of the pathogen, which is necessary for the development of biotechnology of veterinary drugs against ASF in Ukraine.

To improve anti-epizootic measures in the near future, in our opinion, special attention should be paid to info about the relative short-term clinical status of convalescent pigs in terms of viral load of low-virulence variants of the 1st genotype ASF virus [51, 52]. Recent literature data indicate a possible role in these processes of just associative infections and microflora of certain species, i.e., the associative activity of

the ASF agent [47, 52, 54]. It should be emphasized that the data obtained by us allow one to improve the selection of disinfectants with the required virocidal properties for ASF countermeasures as well as to improve the procedure of quality control of disinfection, which is currently counted as the most effective measure to eradicate and prevent this disease in pigs [54] and pork processing [55]. Being developed on the basis of the above data, standard operating procedures meet the international requirements for the use in ASF countermeasures «mainly those disinfectants that have been tested in control trials with local field strains of the agents» [56—58]. Taking into account the circulation of the pathogen of ASF on the territory of Ukraine, which became especially active after the attack of the Russian Federation on Ukraine in 2014, as well as the growing threat of agroterrorism under current conditions, it is advisable to use the developed approaches to control the quality of anti-epizootic and forensic veterinary measures, especially within the framework of agricultural export programs of Ukraine.

Conclusions. Low-cost operational procedures have been developed that allow the use of a vivarium of laboratory animals for ASF bioassay and meet principal requirements for scientifically based research in important aspects of experimental ASF epizootology. With their help, confirmation of the heterogeneity of the population of the ASF virus circulating in endemic foci of the Ukrainian Western Podillia was obtained, at least in terms of biodiversity by pathogenicity ($p \leq 0.05$, $n = 25$). The developed methodological approach is suitable for the study of fundamental issues of ASF epizootology, as well as for the development and control of the effectiveness of chemoprophylactic drugs and disinfectants, as well as quality control of anti-epizootic measures against ASF. In particular, it is advisable to use it to support agricultural export programs of Ukraine, as a country that is disadvantaged by ASF.

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ЕКСПЕРИМЕНТАЛЬНА ЕПІЗООТОЛОГІЯ СЛАБОВІРУЛЕНТНИХ ВАРІАНТІВ ВІРУСУ АФРИКАНСЬКОЇ ЧУМИ СВИНЕЙ

Африканська чума свиней (АЧС) залишається актуальною проблемою свинарства України, вирішення якої можливе лише на основі глибоких наукових знань про специфіку рушійних сил епізоотії в її конкретному нозоареалі. Це необхідно для того, щоб спрямувати протиепізоотичні заходи на найбільш уразливу ланку епізоотичного ланцюга в конкретному нозоареалі. **Метою** дослідження була розробка бюджетощадної методичної бази експериментальної епізоотології слабовірулентних варіантів АЧС в Україні, зокрема для вивчення механізмів формування ензоотичних ареалів, контролю якості протиепізоотичних заходів та оцінки ефективності противірусних препаратів проти них в Україні. **Методи.** Експериментально-епізоотологічні дослідження з адаптації поросят-сисунів до умов їх утримання у віваріях лабораторних тварин з рівнем біозахисту BSL-3 (Патент України № 133248 від 25.03.2019) проводились на лабораторній базі Одеської філії ННЦ «ІЕКВМ». Усі процедури з інфекційно-активними біологічними матеріалами проводилися в чинному порядку в модулі BSL-3, побудованому та сертифікованому за сприяння Уряду США в

УкрНДПЧІ ім. І.І. Мечникова МОЗ України (Одеса). Як тест-вірус використовувався штам збудника АЧС «IECVM/Тернопіль/2017» (інфекційна активність 4,0—7,5 lg $ГАдО_{50/см}{}^3$), що циркулював на території Тернопільської області у 2017—2020 роках. Наявність низьковірулентних варіантів збудника АЧС у досліджуваних зразках визначали біопробою на поросятах-сисунах з подальшими трьома послідовними пасажами на стабільній лінії Vero клітин нирок зеленої мавпи. Ідентифікацію виділених клонів вірусу АЧС проводили методами та діагностикумами, рекомендованими Мануалом МЕБ. **Результати.** Перемежаючі пасажі за методикою «генетичного засновника» розведень 10^{-1} та 10^{-2} штаму вірусу АЧС «ІЕКВМ/Тернопіль/2017» на поросятах (n=20) та в культурі альвеолярних макрофагів свиней, АМС («$ВАЧС^{АМС}$»), дозволили ідентифікувати високо-, помірно- та слабовірулентні варіанти/клони в складі його популяції. Перевірка за допомогою біопроби на поросятах-сисунах (n=5) кластеру слабовірулентних клонів збудника, стабілізованих у культурі клітин Vero («$ВАЧС^{Vero}$»), показала, що після внутрішньочеревного зараження в дозі 4,25 lg $ГАдО_{50/см}{}^3$ він здатний викликати лише нелетальну інфекцію (термін спостереження 2 тижні) з максимальною добовою ректальною температурою 39,4±0,22 ºС і тривалістю лихоманки в середньому 1,6±0,14 діб (n=5 з 5 сисунів). Кластери клонів більш вірулентних («$ВАЧС^{АМС}$») за аналогічних умов викликали виключно летальну інфекцію сисунів з максимальною температурою 40,7±0,37 °С та тривалістю лихоманки в середньому 3,9±0,27 діб (17 з 20 поросят). Слабовірулентні клони ідентифіковали методом прямої імунофлюоресценції в мазках-відбитках легень та селезінки зазначених клінічно здорових поросят на 14 і 17 день після зараження (п.з.); їхні антигени візуалізували в моношарі клітин Vero на скельцях (доза зараження близько 0,01 lg $ГАдО_{50/см}{}^3$) непрямим імунопероксидазним методом через 48 год. п.з; вони викликали «рихлу» гемадсорбцію інфікованих клітин Vero, а їхні віріони мали характерний для асфарвірусу вигляд та розмір (ікосаедри діаметром 210—220 нм). Отримані дані слугували основою аналізу механізму поширення збудника АЧС в ензоотичному осередку Західного Поділля («опільському»), а також розробки регламентів малобюджетного контролю якості протиепізоотичних заходів та оцінки дії противірусних засобів проти АЧС (дезінфектантів та хіміопрепаратів). **Висновки.** Розроблено бюджетощадні операційні процедури, які дозволяють використовувати віварій лабораторних тварин для біотестів на АЧС і відповідають вимогам до доказових досліджень в базових напрямках експериментальної епізоотології АЧС. За їхньою допомогою отримано підтвердження гетерогенності популяції вірусу АЧС, що циркулює в ендемічних осередках Західного Поділля України (p≤0,05, n=25). Розроблений методичний підхід є придатним як для вивчення фундаментальних питань епізоотології АЧС, так і для контролю якості протиепізоотичних заходів та засобів проти АЧС. Зокрема, його доцільно використовувати для посилення біозахисту агроекспорту України як країни, що перебуває в несприятливому становищі щодо АЧС.

Ключові слова: *вірус африканської чуми свиней, біорозмаїття, вірулентність, біопроба, поросята-сисуни, агроекспорт, біозахист.*