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BIOLOGICAL PROPERTIES OF AFRICAN SWINE FEVER VIRUS ASFV/Kaliningrad 17/WB-13869

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Abstract

African swine fever (ASF) is a contagious viral disease of domestic pigs and wild boars of all ages and breeds. To date, the infection is endemic in many European and Asian countries including the Russian Federation. Previously ASF virus isolated and studied by Russian scientists were characterized as highly virulent, with 100 % mortality and assigned to genotype II. However, data on the detection of ASFV with reduced virulence and mortality were later reported, thus further analysis of modern isolates is of high importance. In this work, for the first time, we report the biological properties of the ASF virus (named ASFV/Kaliningrad 17/WB-13869) isolated on the territory of the Kaliningrad region of the Russian Federation. The bioassay was carried out on six large white breed pigs (*Sus scrofa domesticus* L.) weighing 15-20 kg, the experiment was performed in a BSL-3 animal facility at the Federal Center for Animal Health (FGBU ARRIAH). Pigs Nos. 3-6 were infected intramuscularly with ASFV/Kaliningrad 17/WB-13869 (genotype II serotype 8) that was isolated from the tubular bone of a wild boar carcass (Bagrationovskiy District, Kaliningrad Province) at a dose of 10 HAD/head. Two uninfected pigs (Nos. 1, 2) were kept in-contact with the infected ones. Clinical signs and body temperature of experimental animals were registered daily. The presence and severity of clinical signs and pathological changes were expressed quantitatively (the sum of scores for a number of indicators). Clinical score was based on assessment of body temperature, weight, behavior, appetite and water consumption, the state of the digestive and respiratory systems, skin and mucous membranes, the presence of nasal discharge and vomiting. Pathological anatomical autopsy assessed changes in the organs of the spleen, kidneys, liver, lung, submandibular and mesenteric lymph nodes. Points were assigned on a scale of severity of recorded signs from 1 to 3 (the most severe). Blood sampling (5.0 cm^3 from each animal) was carried out until the death of pigs on the 0th, 3rd, 6th, 10th, 13th and 19th days after the start of the experiment. Samples taken from dead animals included; spleen, kidneys, liver, lung, submandibular and mesenteric lymph nodes. Samples and blood were used to prepare 10 % suspensions in sterile saline using an automatic homogenizer, then centrifuged at 400 g (Sigma Laborzentrifugen GmbH, Germany) for 2 min. The resulting supernatant was used for DNA extraction. Blood serum samples were tested for the presence of Anti-ASFV antibodies using ELISA test systems INgezim PPA Compac (Ingenasa, Spain) and ID Screen (IDvet, France), and immunoperoxidase method (IPM). Real-time PCR detected ASFV genome starting from the 3rd day after infection, while IPM detected anti-ASFV antibodies 1-2 days before the death of infected animals, no anti-ASFV antibodies were detected in the serum of animals by ELISA test systems throughout the experiment. Maximum clinical score was registered in animals with a sub-acute form of the disease (21 and 35 points, respectively), while minimum with hyper-acute disease form (6 and 8 points). As a result, ASFV/Kaliningrad 17/WB-13869 virus isolate was characterized as highly virulent, capable of causing ASF in pigs in forms from hyperacute to subacute with mortality up to 100 % of infected and contact animals, which is similar to the clinical picture caused by ASF virus isolates from the Russian Federation in 2007-2018.

Keywords: African swine fever, wild boars, bioassay, laboratory diagnostic methods, clinical signs, pathological changes

African swine fever (ASF) is a contagious viral disease of wild boars and domestic pigs of all ages and breeds [1-3]. To date, the infection is widespread in many countries in Europe and Asia, including the Russian Federation [4]. The causative agent of ASF is a DNA-containing arbovirus of the genus *Asfivirus* (family *Asfarviridae*), which has 10 serotypes identified in the hemadsorption delay test (HAD), and 24 genotypes identified by sequencing the variable C-terminus of the *B646L* gene, encoding the capsid protein vp72 of the pathogen [5-8]).

Russian scientists have studied in detail a number of ASF virus genotype II isolates from pigs and wild boars from various regions of Russia. Thus, isolates have been described that cause from hyperacute to subacute forms of the disease, with 100% lethality for infected animals, the death of which occurred in the period from 3 to 17 days after infection (d.a.i.) [9-12]. In addition to highly virulent isolates, those with reduced virulence have been described with a lethality up to 50% (isolate Lipetsk 12/16) and the period of death after infection within a wider range (5-30 d.a.i.) [13-15].

Similar results were reported abroad on identification of virus variants with reduced virulence, causing from acute to asymptomatic forms of the disease [16-22]. Special attention is paid to the discovery of non-hemadsorbing variants of the virus genotype II Lv17/WB/Rie1 (Latvia, 2017) [23], HeB/Q3/20 and HLJ/HRB1/20 (PRC, 2020) [19], and variants of genotype I HeN/ZZ-P1/21 and SD/DY-I/21 (PRC, 2021) [24].

Reports of ASF virus isolates with reduced virulence which were found in Russia and neighboring countries suggest the formation of endemic zones. This complicates the early diagnosis of the disease, posing additional problems in combating infection, and requires further study of the biological properties of the isolates [13, 25-27].

In this work, the biological features of the ASF virus isolate circulating in the Kaliningrad Province of the Russian Federation are established for the first time.

Our goal was to study properties of the ASFV/Kaliningrad 17/WB-13869 isolate.

Materials and methods. The work was carried out in 2018-2020 at the vivarium complex of the Federal Center for Animal Health Protection (ARRIAH) which provides for work with pathogens of pathogenicity groups 2-4. The test was carried out on pigs (*Sus scrofa domesticus* L.) from a pig farm free from major infectious diseases (Vladimir Province) in strict accordance with interstate standards for the maintenance and care of laboratory animals adopted by the Interstate Council for Standardization and Metrology and certification, as well as in accordance with the requirements of Directive 2010/63/EU of the European Parliament (EU Directive 2010/63/EU) and the Council of the European Union of 22.09.2010 on the protection of animals used for scientific purposes. Six Large White pigs weighing 15-20 kg, before the start of the experiment, were kept in quarantine for 7 days to assess their clinical condition.

Pigs Nos. 3-6 were infected with cultural material containing hemadsorbing ASF virus genotype II serotype 8 (isolate ASFV/Kaliningrad 17/WB-13869) from the tubular bone of a dead wild boar, discovered on November 7, 2017 in the Krasnoarmeyskoye village (Kaliningrad Province, Bagrationovsky District) (strain ASF/Kaliningrad-10/17, isolated and deposited by ARRIAH).

The virus-containing suspension was intramuscularly administered at a dose of 10 hemadsorption unins (HADU) per animal. Two uninfected pigs (Nos. 1, 2) were kept together with infected ones to assess possible contact infection. Biological tests and assessment of clinical signs and pathological changes were carried out in accordance with the ARRIAH methodological recommendations and guidelines [28, 29].

Clinical signs were visually monitored daily, the body temperature of each pig was measured rectally. The presence and severity of clinical signs and pathological changes were expressed quantitatively (sum of points for a number of indicators). Body temperature, fatness, behavior, appetite and water consumption, the state of the digestive and respiratory systems, skin and mucous membranes, nasal discharge and vomiting, and incubation period indicators were assessed. During the pathological autopsy, changes in organs were examined. Points were assigned on a scale of recorded sign severity from 1 to 3 (the most severe).

Blood samples (5.0 cm^3) were collected individually in test tubes with a coagulation activator until the death of pigs on days 0, 3, 6, 10, 13 and 19 after the start of the experiment. Blood samples were separated into clot and serum. On the day of animal death, pathanatomical autopsy of the spleen, kidney, liver, lung, submandibular and mesenteric lymph nodes was performed, one sample of each organ from each dead pig). The samples were examined immediately or stored at –70.0 °C.

Blood sera were used without pretreatment. Blood clots and organs were homogenized (an automatic homogenizer, QIAGEN GmbH, Germany) in sterile saline to a 10% suspension, then centrifuged at 400 g (Sigma Laborzentrifugen GmbH, Germany) for 2 min. DNA was extracted from the resulting supernatant.

To assess specific antibodies to the ASFV in blood serum, enzyme-linked immunosorbent assay tests INgezim PPA Compac (Ingenasa, Spain) and ID Screen, African Swine Fever Indirect, Screening Test (IDvet, France) were used in accordance with the manufacturers' instructions. Additionally, immunoperoxidase test (IPT) was used as guided by the ARRIAH methodological recommendations [30]. INgezim PPA Compac is a solid phase competitive enzyme-linked immunosorbent assay (SP-ELISA) based on the ASFV vp72 protein and monoclonal antibodies to it. ID Screen is an indirect version of SP-ELISA using the p32, p62 and p72 antigens of the ASFV. The essence of IPT is to identify specific antigen-antibody complexes resulted from the interaction of antigens of the virus replicating in an infected cell culture, the ASFV and the antibodies to ASFV.

DNA was isolated by phenol-chloroform extraction using the DNA-Sorb-B kit (Central Research Institute of Epidemiology of Rospotrebnadzor, Russia). RT-PCR was performed using the Test System for Detection of the African Swine Fever Virus Genome by Real-Time Polymerase Chain Reaction (ARRIAH, Russia) according to the manufacturer's instructions. The number of genome copies (g.c.n.) of the ASF virus in the blood and organs of animals was calculated accoding to recommendations [31]. All samples were examined once in one repetition.

Data processing and plotting were carried out using GraphPad Prism 8.0 software packages (https://www.graphpad.com/) and Microsoft Excel (https://www.microsoft.com/ru-ru/). Means (M) and standard errors of means (±SEM) were calculated.

Results. After the appearance of the first clinical signs, the disease lasted from 4 to 7 days in infected animals and from 2 to 14 days in contact animals (Table 1), which is typical for hyperacute, acute and subacute ASF [32, 33].

Throughout the entire observation period, clinical signs characteristic of ASF were recorded in the animals. Until death, contact pig No. 1 had no symptoms other than an increase in body temperature above the physiological norm (40.0 °C) and ataxia. In infected animals (Nos. 3-6) and in contact pig No. 2, clinical signs characteristic of ASF appeared. These signs were loss of appetite (up to complete refusal of feed and water), damage to the nervous system (from

asthenia to areflexia), respiratory system (from lung to severe dyspnea) and digestive system (registration of diarrhea from mild to dehydration), cyanotic zones (up to 15% of the skin surface), conjunctival hyperemia (from moderate to severe with serous-purulent exudate) (Fig. 1).

1. Assessment of clinical signs (score) **of the course of African swine fever in Large White pigs (*Sus scrofa domesticus* L.) infected with isolate ASFV/Kaliningrad 17/WB-13869** (tests in the vivarium of the Federal Center for Animal Health, 2018)

Group	Pig No.	Days after challenge																			
		0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Contact	1														3	6	†				
	2							4	4	5	4	8	8	6	7	11	14	17	19	20	21 †
Infected	3	ϟ				4	5	6	7	10	12	13	†								
	4	ϟ					6	4	5	9	11	14	†								
	5	ϟ						6	5	5	12	†									
	6	ϟ						5	5	8	9	15	†								

Note. ϟ — date of infection, † — date of death.

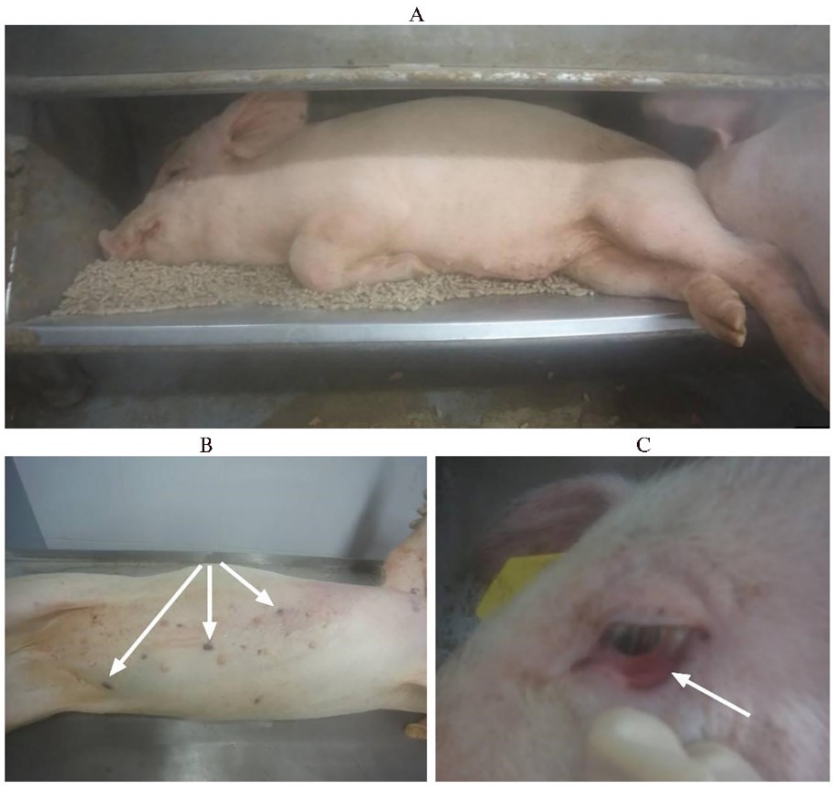

Fig. 1. Clinical signs of African swine fever in a Large White pigs (*Sus scrofa domesticus* L.) infected with isolate ASFV/Kaliningrad 17/WB-13869: A — ataxia, decreased response to external stimuli, B — foci of skin necrosis, C — hyperemia of the conjunctiva (tests in the vivarium of the Federal Center for Animal Health, 2018).

Thus, in total, pig No. 1 (superacute form) scored 6 points, infected pigs No. 3-6 (acute form) scored 12-15 points, and pig No. 2 (subacute form) scored 21 points.

An increase in body temperature (> 40.0 °C) was recorded in the infected animals starting from 4-6 days after the challenge, while in contact pigs No. 1 and No. 2 from 13 and 6 days, respectively (Fig. 2). In infected pigs Nos. 3, 4, viremia was recorded starting from day 3, in pigs Nos. 5, 6 from day 6. The maximum genomic load reached 7.8 g.c. per ml ($\log_{10}$).

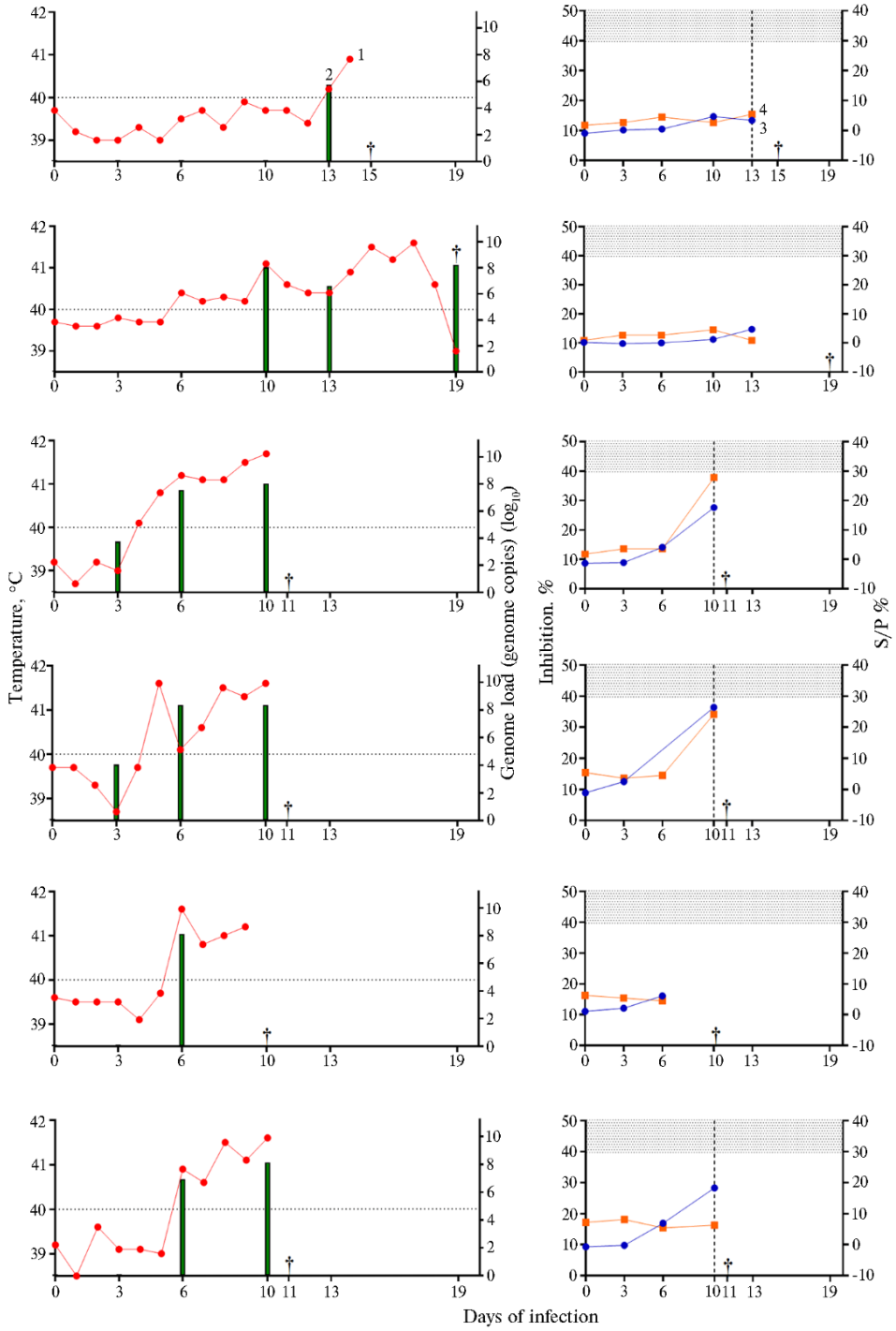

Fig. 2. Body themperature (1)**, genomic load** (2) **and blood sample assay by ELISA test** (3) **and immunoperoxidase test** (IPT) (4) **in Large White pigs (*Sus scrofa domesticus* L.) infected with the African swine fever virus isolate ASFV/Kaliningrad 17/WB-13869:** from top to bottom — pig No. 1, No. 2, No. 3, No. 4, No. 5, No. 6, respectively. The horizontal dotted line along the temperature axis marks the border of the physiological norm (40.0 °C); vertical dotted lines indicate positive results (IPM method); † — death dates (tests in the vivarium of the Federal Center for Animal Health, 2018).

Specific antibodies to the ASFV were not detected by TF-ELISA test. The degree of inhibition did not exceed 36.4% for the Ingezim PPA Compac

test system (samples with < 40% inhibition are considered negative). When using the ID Screen, African Swine Fever Indirect, Screening Test system, the maximum S/P% value (the ratio of the optical density of the test sample to the optical density of the positive control) was 27.8% (samples with an S/P < 30% are considered negative). Moreover, in simultaneous laboratory IPT assay, samples taken 1-2 days before the death of one contact (No. 1) and three infected animals (Nos. 3, 4 and 6) were positive for the specific antibodies.

2. Assessment of pathological changes in the Large White pigs (*Sus scrofa domesticus* L.) infected with the African swine fever virus isolate ASFV/Kaliningrad 17/WB-13869 (tests in the vivarium of the Federal Center for Animal Health, 2018)

Pig No.	Lungs			Heart		Spleen		Lymph nodes			Liver		Kidneys			Bladder	Transudate		Σ
	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV	XV	XVI	XVIII	XIX	
1	-	-	1	2	-	1	1	1	–	–	–	–	1	–	1	–	–	–	8
2	2	2	1	2	1	3	2	3	2	2	2	2	2	2	2	2	2	1	35
3	1	1	1	1	1	2	2	2	1	–	1	1	1	1	1	1	1	–	19
4	2	2	1	1	2	2	2	2	2	1	–	–	2	2	2	1	2	1	27
5	1	1	1	1	1	2	2	1	1	–	1	-	1	–	1	1	1	–	16
6	2	3	1	2	1	1	1	1	–	1	1	1	1	1	1	1	1	–	20
Σ	8	9	6	9	6	11	10	10	6	4	5	4	8	6	8	6	7	2	125

N o t e. I — edema, II — pneumonia, III — hemorrhages under the pleura; IV — hemorrhagic diathesis, dystrophy, V — transudate in the pericardial cavity; VI — blood filling, VII — splenomegaly; VIII — submandibular lymph nodes, IX — mesenteric lymph nodes, X — inguinal lymph nodes; XI — hepatopathy, XII — bile ducts; XIII — hemorrhagic diathesis in the cortex and medulla, XIV — subcapsular hemorrhages, XV — hemorrhages in the renal pelvis; XVI — hemorrhagic diathesis in the mucous membrane, XVIII — chest cavity, XIX — abdominal cavity; Σ — sum of points. Dashes mean that the corresponding pathoanatomical changes were not identified.

After death, an autopsy of each animal was performed. Of the characteristic pathoanatomical changes, we noted, in particular, blood filling of the spleen (11 points), splenomegaly (10 points), enlargement of the submandibular lymph nodes (10 points), and pneumonia (9 points) (Table 2).

The same as for clinical signs, the severity of pathological changes depended on the form of the disease. Thus, the lowest score was in pig No. 1 (8 points), while the highest was in pig No. 2 (35 points).

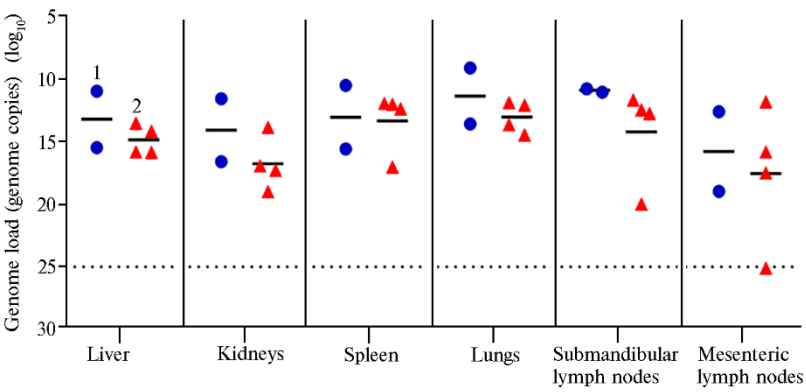

Fig. 3. The genomic load in the organs of dead Large White pigs (*Sus scrofa domesticus* L.) infected with the African swine fever virus isolate ASFV/Kaliningrad 17/WB-13869: 1 — contact animals, 2 — infected animals (tests in the vivarium of the Federal Center for Animal Health, 2018).

The ASFV genome was detected in all organs (Fig. 3) and the genomic load averaged 6.23±0.16 g.c. per ml ($\log_{10}$).

It should be noted that the identified biological properties of the isolate ASFV/Kaliningrad 17/WB-13869 are comparable with those of hemadsorbing isolates of the ASFV genotype II detected in the Russian Federation (Stavropol 01/08, Pskov-Yashkovo, ASFV/Primorsky 19/WB-6723 etc.) [22, 32, 34], the

Republic of Poland (Pol16/DP/OUT21) [17], the People's Republic of China (Pig/HLJ/18) [16], Georgia [2] and Armenia [1], which have 100% lethality for pigs. In our research, the time from infection with the studied isolate to an increase in body temperature above the physiological norm (40.0 °C) and the ASFV genome detection in the blood coincided with those for other highly virulent isolates and amounted to 4-6 days and 3-6 days, respectively. Other clinical signs of ASF were also noted, such as loss of appetite, damage to the nervous and digestive systems, appearance of cyanotic zones, and hyperemia of the conjunctiva [13, 16, 17, 32, 33].

In contrast to the ASFV genome that was recorded in the blood of infected pigs from the first assessment until the animal death, we found specific antibodies to the pathogen only when examining blood serum from three pigs infected intramuscularly and one contact pig using IPT. Comparable data were obtained by C. Gallardo et al. [17[. Using IPT, these authors detected specific antibodies to the ASFV in 83.3% of serum samples 7 d.a.i. with ASFV isolate Pol16/DP/OUT21 (Poland, 2016), while in SP-ELISA, only one contact pig at 16 d.a.i. was ASFV positive. In the work of A. Pershin et al. [13] specific antibodies were detected in 19.3% of blood serum from pigs infected with various ASFV isolates, 13 in total, sampled in 2013-2018 in the Russian Federation. The first positive results were noted from 14.14±2.00 d.a.i. However, A.R. Shotin et al. [22] did not detect specific antibodies to the ASFV whrn testig the ASFV/Primorsky 19/WB-6723 isolate (Primorsky Krai, 2019). The data obtained may be a consequence of the rather rapid death of animals, none of them lived for more than 7 days (for infected animals) and 14 days (for contact animals) from the moment when the first clinical signs of ASF appeared [35]. Another reason may be higher sensitivity of IPN compared to SP-ELISA [36-38].

The severity of pathological changes, as well as the severity of clinical signs, correlated with the duration of the disease. Characteristic lesions for ASF described by domestic and foreign researchers for ASFV isolates from China (Pig/HLJ/18) [16], Poland (Pol16/DP/OUT21) [17], Vladimir (Shikhobalovo 10/13) [9], Ryazan (Ryazan-Sapozhkovo/2016) [10], Novgorod (Novgorod-Okulovo) [11] regions, Primorsky Krai [22] and other regions of Russia, were observed in the lungs, heart, spleen and other organs.

Thus, the results of the studies allow us to characterize the isolate of the African swine fever virus (ASF) ASFV/Kalinin-grad 17/WB-1386 as highly virulent, capable of causing ASF in pigs, from hyperacute to subacute, with the death rate up to 100% of infected animals and those having a direct contact. Despite the data obtained, there is a possibility of survival of some animals infected, including with highly virulent isolates of the ASF virus, as well as the possibility of changing the biological properties of already circulating variants of the pathogen. This requires the additional use of direct (RT-PCR, virus isolation) and indirect (SP-ELISA, IPT) research methods to identify the ASF virus and/or its genome and specific antibodies to this infectious agent.

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