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Research article

CHARACTERISTICS OF AFRICAN SWINE FEVER VIRUS ISOLATED FROM DOMESTIC PIGS AND WILD BOARS IN THE RUSSIAN FEDERATION AND SOUTH OSSETIA

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The article presents the results of a comparative study of the biological, molecular and genetic characteristics of African swine fever virus (ASFV) isolates, obtained from domestic pigs and wild boars in the Russian Federation and South Ossetia from 2016 to 2018. The studied isolates caused the death of pigs manifesting, as a rule, signs of an acute or subacute form of the disease when using various methods of infection including intramuscular, direct contact, intranasal and oral routes. The virus was hemadsorbing, belonging to serotype 8 and genotype II, and accumulated in the blood with a titer of 6.5 to 7.5 lg HAU_{50}/cm^3. The ASFV isolates circulating in the central region of Russia were found to have an insertion of 10 base pairs in the intergenic region I73R/I329L. However, the ASFV isolated in the Irkutsk region and South Ossetia, as well as Georgia 2007/1 (FR682468.1), lacked this insertion.

Key words: African swine fever, serotype, genotype, infectious activity, antibody

INTRODUCTION

In 1957, the first African swine fever (ASF) outbreak was detected in Europe, initially in Portugal on pig farms located near the international airport of Lisbon and then, in 1960, in Spain. In the following years, the disease outbreaks were recorded in France (1964, 1967, 1974), Italy (1967, 1978, 1984), Malta (1967), Belgium (1985), the Netherlands (1986), and also in the USSR (1977) [1,2]. African swine fever has caused enormous economic lossess to these countries due to the implementation of strict anti-epizootic measures.

The beginning of the recent ASF epizootic in Europe was associated with the introduction of the infectious agent in Georgia in 2007. In the same year, the disease

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entered Russia, namely Republic of Chechnya, with wild boars. Based on its nucleotide structure, the African swine fever virus (ASFV) circulating in Georgia was found to belong to genotype II, and, in this respect, was homologous to the viruses isolated in Mozambique and on Madagascar Island [3-5]. According to the OIE and the Federal Service for Veterinary and Phytosanitary Supervision of the Russian Federation, over a period from 2007 to 2018, as many as 11,829 ASF outbreaks were recorded in 17 countries of Europe and Asia, including 2,903 ones registered among domestic pigs and 8,926 in wild boars, the infection having been recorded mainly in wild boars in Hungary, Lithuania, Latvia, Estonia and Poland [6,7]. In 2019 ASF was introduced in Serbia, affected 18 holdings before the outbreak was contained, and was not confirmed in wild boars so far [8].

In the wild, ASF is most often transmitted through direct contact (oronasal route) and/or via the alimentary tract by means of contaminated feed, water, fomites, etc. It can also be passed through biting and mechanical vectors, i.e. through argasid ticks of the genus *Ornithodoros* on the African continent and in Sardinia, as well as stable flies (*Stomoxyscalcitrans*), in which the virus remains infectious for 48 hours [9,10].

The main portals of ASFV entry are the respiratory tract, the oral, the nasal and/or the ocular mucous membrane, and damaged skin [4]. The disease can be peracute, acute, subacute, chronic, and, in the enzootic zones, asymptomatic [11-13].

In the Russian Federation, the European Union and China, ASFV virulent isolates currently circulating basically give rise to an acute or a subacute form of the disease [14-16].

Until 2013, the disease was manifested in the acute form with the characteristic clinical signs and pathological changes both among domestic pigs and wild boars in the Russian Federation. In 2014, an ASFV "Kaluga-2014" was isolated in the Russian Federation from wild boars which caused the death of domestic pigs, without typical clinical symptoms and pathomorphological changes [17]. At the same time, in Estonia a low-virulent ASF virus was isolated that turned out to be a deletion mutant and resulted in the recovery of a part of the pigs that survived the experimental infection [18-22].

Considering the long-term circulation of the ASF virus in the Russian Federation and South Ossetia, this research was aimed to study the biological and molecular genetic characteristics of the disease agents isolated from 2016 to 2018 both from domestic pigs and wild boars.

MATERIALS AND METHODS

ASF virus isolates

We used isolates of the ASF virus detected from 2016 to 2018 when carrying out diagnostic and monitoring surveillance of domestic pigs in Orel (Orel/Shablykinsk/2016), Nizhny Novgorod (Diveyevo/Nizhny Novgorod/2016), Irkutsk

(Irkutsk-2017) and Saratov (Saratov-2017) regions, in the Republic of Tatarstan (Tatarstan-Sosnovka/2016), and South Ossetia (G-2018), as well as wild boars in Kabardino-Balkaria (Baksansky/KBR/2016), Pskov (Pskov-Yashkovo/2016), Moscow (St. Pokrov/O-3/2016) and Vladimir (Vladimir-Vyazniki/2017) regions.

Animals

Pigs of Yorkshire breed live weight between 25 and 30 kg from the Animal Preparation Sector, Federal Research Center for Virology and Microbiology (FRCVM), Volginsky, the Russian Federation, were infected with ASF virus intramuscularly, orally, intranasally, and through contact using at least 2 or 3 animals for each method. For every strain and group we also used 1 animal as a control, injecting it 1 ml of phosphate-buffered saline.

The intramuscular infection was carried out through injection of organ (namely, lymph nodes and/or spleen) suspension into the middle third part of the neck at a dose of 3.0 lg HAU_{50}.

The contact infection was carried out by jointly keeping the ASF-affected pigs and the intact animals for 10 to 12 hours, the latter ones being transferred then to separate quarters.

The intranasal infection was carried out by inoculation of infectious material at a volume of 5.0 cm^3, a dose of 3.0 lg HAU_{50} with a syringe through a silicone tube in the nose.

The oral infection was carried out by feeding the pigs with forage containing a mixed spleen and lymph node 10% suspension or virus-containing blood dissolved in water, at a dose of 7.0 lg HAU_{50}.

Animals were monitored for 35 days, each animal was necropsied for detection of pathological changes.

Cell cultures

Swine leukocyte (SL) culture was prepared and ASFV identification in hemadsorption and autohemadsorption tests was carried out in accordance with GOST 28573-90 [23, 24]. The ASFV infectious activity was determined by titration in a SL primary culture grown in 24-well plates. The virus titers were calculated using the Kerber method in Ashmarin modification, and expressed in lg HAU_{50}/cm^3 [25].

PCR

The ASF virus genome was detected by real-time polymerase chain reaction (real-time PCR) in CFX 96 amplifier (BioRad, the USA) using a Taq-man probe (Z1(int)p30 5’-(FAM)TACTGTT(RTQ1)AAGTATGATATTGTGA(BHQ1)-3’), and oligonucleotide primers (F1 p30 5’-GTTACGACCGCTATAAAAACA-3’ and R1 p30 5’-TTCCATTCTTCTTGAGACCTG-3’) flanking a fragment of gene CP204L.

The virus total DNA was isolated using a kit "DNA-sorb" (Interlabservice, Russia) according to the manufacturer's instructions [26].

Antibody detection

The virus-specific antibody detection in the blood sera and 10% organ suspensions of dead animals was performed using enzyme-linked immunosorbent assay (ELISA), indirect fluorescent antibody (IFA) test and/or immunoblotting. The organ suspensions were obtained by homogenizing 1 g of tissue in 9.0 cm^3 of sterile saline. The resulting 10% homogenates (w/v) were frozen twice at -40±0.5 °C, thawed at room temperature, and clarified by centrifugation (3000 rpm, 15 min). The supernatants were stored at -40±0.5 °C and used for serological reactions.

The IFA test was performed using a commercial kit (FRCVM, Russia) for differential immunofluorescent diagnosis of African swine fever, classical swine fever, and Aujeszky's disease. The results were recorded by fluorescent microscopy (Nikon Co. microscope, Japan).

ELISA was carried out using a commercial set of ASF-ELISA Ab/Ag (FRCVM, the Russian Federation).

The immunoblotting assay was carried out with a test system for African swine fever serodiagnosis using the immunoblotting technique manufactured by FRCVM [27, 28]

The isolates were typified according to the "Guidelines for African swine fever type-design practice through hemadsorption inhibition test" using reference sera and ASFV reference strains of serotypes I-IX, which were obtained from the National Bank of Microorganisms (NBM), FRCVM [29].

Phylogenetic analysis

For genotypic identification of the virus isolates, the DNA sequences were analyzed using a program BLASTN [30]. The identity of the sequences was determined based on the statistical significance of their matches. The additional sequence analyses were performed through alignment with the known homologous sequences of ASF virus genes available in the GenBank database using a program ClustalX.

RESULTS

Clinical and necropsy findings

All the pigs infected using various methods of administration manifested the ASF clinical signs basically characteristic for the acute or the subacute form of the disease. Some isolates were not tested with all routes of infection due to the lack of animals, and will be tested during the next experiments. We observed depression, hyperthermia up to 41.5-41.9 °C, painful breathing and refusal of food. However, in most cases

diseased animals did not exhibit any pronounced cyanosis of the skin, or vomiting and/or bloody flux were neither observed.

In the intramuscularly infected pigs that died with the signs of an acute form of ASF, the incubation period was 2 to 6 days. The disease duration ranged from 3 to 6 days, with slight cyanosis of the skin in the ear and/or perineal areas being observed or were altogether absent (isolate G-2018) (Fig. 1a), also paresis and paralysis of the hind limbs 1 or 2 days before death were seen. Results of challenges are shown in Table 1.

Table 1. Characteristics of course of the disease as seen in pigs infected with various ASFV isolates using different administration routes

No	Designation of isolate	Route of infection	Characteristics of the infection			
			Incubation period (days)	Disease duration (days)	Time of death (days)	Form of the disease
1	Orel/Shablykino/2016	i/m	2.3	5.6	7.9	acute
2	Diveyevo/Nizhny Novgorod/2016	i/m	2.3	6	8.9	acute
3	Baksansky/KBR/2016	i/m	3.4	6.8	10.11	acute
		contact	5	7	12	acute
			7	8	16	s/acute
4	Tatarstan-Sosnovka/2016	i/m	3	3.4	6.7	acute
			1	3	4	p/acute
		contact	4.6	7.8	12.13	acute
5	Pskov-Yashkovo/2016	i/m	2	4.6	6.8	acute
		peroral	6.9	8.9	16.17	s/acute
6	St. Pokrov/O-3/2016	i/m	3.5	4.5	7.10	acute
7	Irkutsk-2017	i/m	3.4	2.3	5-6	acute
			3	1	4	p/acute
8	Vladimir-Vyazniki/2017	i/m	6	4.5	10.11	acute
		contact	6.9	4-6	10-14	acute
		peroral	6.10	4.6	12.14	acute
			7-14	5-8	16-22	s/acute
		intranasal	8	5	13	acute
			9	6	16	s/acute
9	Saratov-2017	i/m	5	4.5	9.10	acute
		contact	8	5.6	13.14	acute
10	G-2018	i/m	4	3.4	7.8	acute

Note: i/m - intramuscular injection; p/acute – peracute form, s/acute – subacute form.

The fallen animals exhibited splenomegaly (Fig. 1b), serofibrinous and hemorrhagic exudates in the thoracic and the abdominal cavities, slight enlargement of submandibular, mediastinal and/or mesenteric lymph nodes (Fig 1c), and hepatomegaly (Fig 1d). At the same time, the gastric (Fig. 1e) and the portal lymph nodes were swollen up to 2 to 4 times their original size. They were like a blood clot in color and had a flabby consistency, and were observed in all cases except for the ones with a peracute form of the disease. Some of the animals exhibited hemorrhages on the epicardium, and petechiae in the lungs and the kidneys (Fig. 1f). Similar alterations were observed in

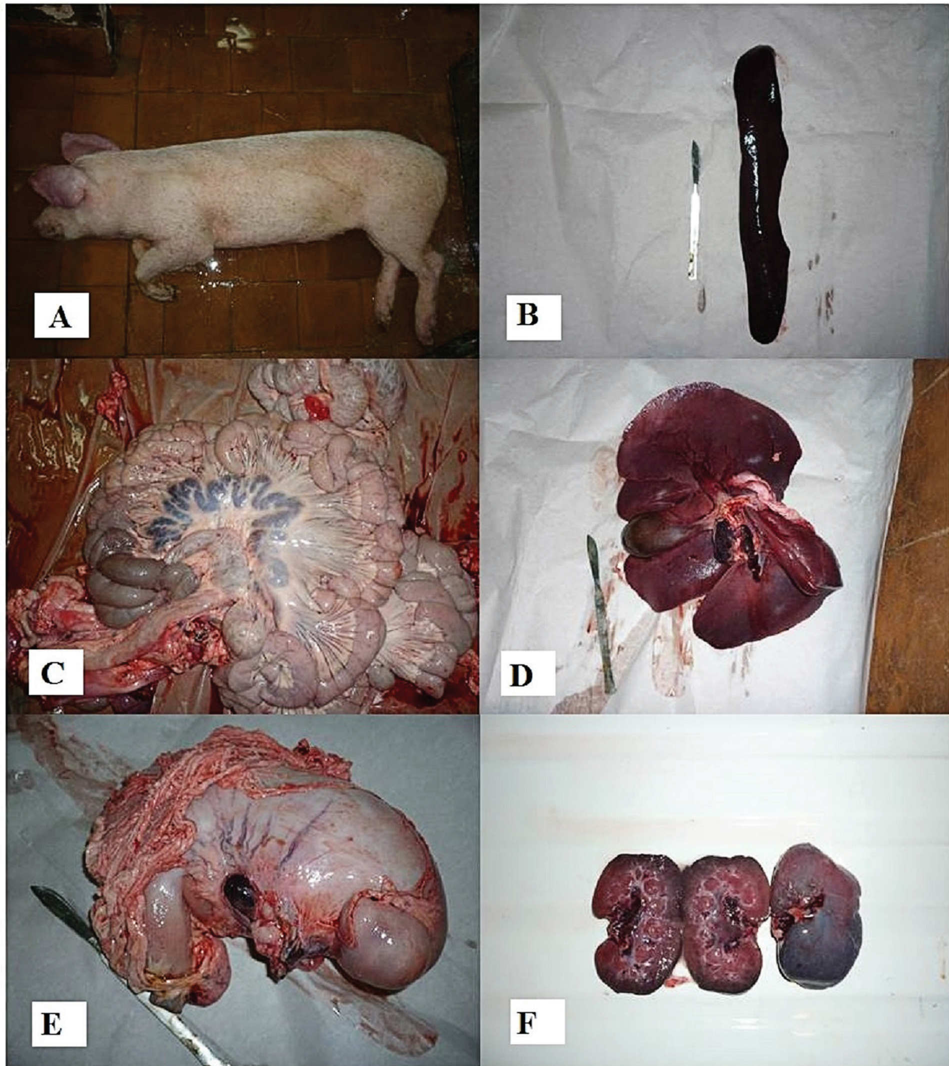

Figure 1. Acute form of ASF infection in pigs: **a)** slight ear cyanosis; **b)** splenomegaly; **c)** enlarged mesenteric lymph nodes; **d)** hepatomegaly; **e)** stomach with enlarged and hemorrhagic gastric lymph nodes; **f)** hemorrhages on the kidneys.

pigs that had died following infection with isolate G-2018, with no abnormalities characteristic of the acute ASF to be seen in the lungs, the heart and/or the kidneys. In pigs infected with isolate Pskov-Yashkovo the characteristic gross lesions were observed only in the gastric and the portal lymph nodes.

In the peracute form of ASF, as seen in pigs infected with isolates Tatarstan-Sosnovka/2016 and Irkutsk-2017, the incubation period and the duration of the disease were 1 to 3 days, the clinical signs being mild. In these animals, 1.5- to 2-fold swollen spleen and serofibrinous exudates in the thoracic and the abdominal cavities were observed. The gastric and the portal lymph nodes were 2 to 3 times swollen and looked like a blood clot.

In the contact-infected pigs, the incubation period ranged from 4 to 9 days, the duration of the disease being 4 to 8 days. The two pigs infected with isolates Vladimir-Vyazniki/2017 and Baksansky/KBR/2016 manifested slight cyanosis of the skin on the ears. The pig died on day 16 (Baksansky/KBR/2016) with diarrhea. The dead pigs showed lymphadenitis of the submandibular, the mesenteric and the mediastinal lymph nodes. The gastric and the portal lymph nodes resembled a blood clot, and were 3 to 4 times swollen. The spleen was red-brown in color, 1.5 to 2 times swollen, and had hemorrhages, and the slightly enlarged and blood-filled liver was dark red in color. The kidneys had petechiae. The pig that died on day 16 had serofibrinous exudate in its thoracic cavity, also the signs of pneumonia were observed.

The pigs intranasally infected with isolate Vladimir-Vyazniki/2017 became ill on day 8 or 9, and fell on days 13 and 16 with the signs of an acute or a subacute ASF form. The fallen pigs had serofibrinous fluid mixed with blood in the thoracic cavity, and dot hemorrhages in the lungs and the mediastinal lymph nodes. The gross lesions as observed in the other lymph nodes, the spleen and the liver were the same as the ones found in pigs following the contact infection. The kidney and the heart had no gross lesions.

The animals orally infected with 10% suspension of lymph nodes and spleen manifested the first clinical signs on day 6 to 9, and those infected with the contaminated water, on day 13 to 14. Among the three animals that were given the infected virus-containing water, one did not get affected and remained clinically healthy for 35 days (the observation period). The duration of the disease as observed in the infected pigs lasted 3 to 9 days with an ill-defined clinical picture. In pigs which died on days 14 to 17, a 1.5- to 2-fold enlargement of the submaxillary, mediastinal, and/or the renal lymph nodes with hemorrhages at the cut surface was noted. The pigs that fell on days 19 and 22 lacked any characteristic pathological changes. They exhibited a slight enlargement of the submaxillary, mesenteric, renal, mediastinal, portal and/or gastric lymph nodes, as well as the spleen and the liver.

Antibody detection

One to two days before the rise in body temperature, hemadsorbing cells were detected in the leukocyte fractions from the blood of the infected animals using an autohemadsorption assay, which suggested ASF virus reproduction. The virus accumulation levels in the blood as observed within this period were 2.5 to 3.5 lg HAU_{50}/cm^3, being as high as 5.0 to 6.5 lg HAU_{50}/cm^3 in the first days of the ASF clinical manifestations. The highest virus accumulation levels reached 6.5 to 7.5 lg HAU_{50}/cm^3 in the blood from animals being in a moribund condition. All the virus isolates studied were the hemadsorbing ones, i.e., they caused hemadsorption in SL primary cells on day 2 to 3 post infection. The virus titers observed in the blood and the organ samples of the dead pigs infected with the test isolates were approximately similar, namely 6.5 to 7.5 for the blood, 5.5 to 6.5 for the spleen, 4.5 to 5.0 for the liver and lymph nodes, and 2.0 to 3.0 lg HAU_{50}/cm^3 for the lungs and kidney.

In the blood sera of the affected pigs, the virus-specific antibody was detectable by ELISA, IFA test or immunoblotting from day 6 post infection. In dead animals the antibodies were found in organ suspensions (spleen, liver, lungs and/or a range of lymph nodes) within the same time period.

The most effective serological method was ELISA that provided virus-specific antibody detection in organ suspensions in 91.4% of cases. For IFA test, the virus-specific antibody detection rate was 47.4%. The low antibody detection rate for immunoblotting (19.7%) is associated with the recommendation of its application for the detection of seropositive animals affected with chronic or asymptomatic forms of the disease in endemic areas [27]. The antibody titers as seen in the tested samples (152 samples) were 1:80 to 1:160 for ELISA, 1:10 to 1:20 for IFA test, and 1:10 for immunoblotting.

The serotype identification of the ASF virus isolates was carried out using the hemadsorption inhibition test. All the isolates under study belonged to ASF virus serotype 8 circulating in Russia and South Ossetia since 2007.

Phylogenetic analysis

Based on the analysis of the sequences of the region I73R/I329L (Fig. 2), we determined that the isolates circulating in the Russian Federation had an insertion TRS+ of 10 nucleotide pairs (TATATAGGAA) and were similar to the isolates found

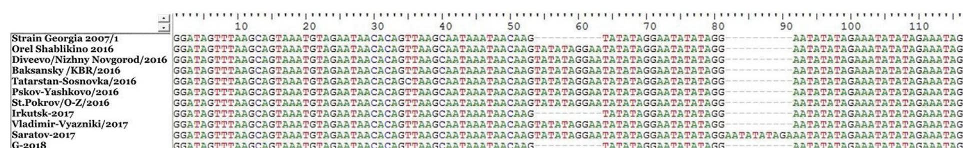

Figure 2. Fragments of sequences of intergenic region I73R/I329L of ASF virus isolates found in the Russian Federation and South Ossetia, aligned with a reference genome Georgia2007/1 (FR682468.1).

in the countries of the European Union. According to the data from nucleotide sequencing and the phylogenetic analysis of gene B646L, all the virus isolates under study belong to genotype II.

DISCUSSION

A long circulation of a virus in wild nature can result in the appearance of low-pathogenic strains, which cause a chronic or asymptomatic course of the disease. In a study by L.Zani with isolate Estonia, it was shown that the mutant strain with the deletion causes the disease with recovery of some pigs [18]. Due to this, testing of different strains can be important for the study of the possible different courses of the disease and better understanding of the role of some ASFV genes (in the case of deletion mutants). Our results show that even for virulent strains different routes of infection can affect the course of the disease, which can be crucial for diagnostic purposes and preventive measures against ASF.

The ASFVs isolated from domestic pigs and wild boars in the Russian Federation and South Ossetia from 2016 to 2018 are virulent, cause animal death at various routes of infection within the time periods typical for an acute, a subacute or a peracute form of the disease, with no complete symptom complex of the clinical signs manifested or pathoanatomical changes observed. The virus is hemadsorbing, it accumulates in the blood of pigs affected at titers of 6.5 to 7.5 lg HAU_{50}/cm^3. In the suspensions of organs collected from pigs dead due to acute ASF, the virus-specific antibody is present and can be detected from day 6 using ELISA, IFA test and/or immunoblotting, the most effective method being ELISA that provided virus-specific antibody detection in 91.4% of cases.

In order to determine new genetic markers that allow for the differentiation of closely related isolates belonging to genotype II, Gallardo et al. [31] developed primers for the intergenic region I73R/I329L using the sequence of strain Georgia 2007/1, and through their use an additional 10-nucleotide insertion was identified in a number of isolates from Ukraine, Belarus, Lithuania and Poland [32]. The presence or the absence of this insertion allows the differentiation of the recent Russian and the European ASFV isolates [33, 34]. The above data indicate that ASF virus isolates taken from boars and/or domestic pigs in the Russian Federation belong to genotype II, variant TRS +. However, TRS – variants of the virus are also present in some regions of Russia. These include Irkutsk-2017 and G-2018, isolated from dead domestic pigs in the Irkutsk region and South Ossetia, respectively. They lacked this insertion and were homologous to Georgia 2007/1 isolate [34]. At the same time, an additional insertion of 10 nucleotides in size was identified in the isolate found in the Saratov region.

Ethics

The animal experiments were approved Institute's Research Ethics Committee and were conducted in animal biosecurity facilities (77.99.03.001.K.000702.0405) Federal Research Center for Virology and Microbiology (FRCVM), Volginskiy, Russia.

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Authors' contributions

MV, AI and SM carried out the experiments on animals. AS and IT performed molecular studies and participated in writing. MV and EP performed the part with cell cultures. VL, MV and AI carried out antibody detection. MV and TS prepared the manuscript. VB and NV participated in the design of the study and critically revised the manuscript. All authors read and approved the final manuscript.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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KARAKERISTIKE VIRUSA AFRIČKE KUGE SVINJA IZOLOVANOG IZ DOMAĆIH I DIVLJIH SVINJA U RUSKOJ FEDERACIJI I JUŽNOJ OSETIJI

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U radu su prikazani rezultati uporedne studije bioloških, molekularnih i genetskih karakteristika izolata virusa, izazivača Afričke kuge svinja (ASFV), dobijenih od domaćih i divljih svinja u Ruskoj Federaciji kao i iz regiona Južne Osetije u periodu od 2016. do 2018. godine. Izolati koji su ispitivani, izazivali su uginuća svinja pri čemu su se simptomi manifestovali, po pravilu u skladu sa akutnim ili subakutnim tokom oboljenja posle primene različitih načina inokulacije prilikom veštačke infekcije uključujući intramuskularni, direktni kotakt, intranazalini i oralni pri čemu inficirane životinje nisu pokazivale tipične kliničke i/ili patološke promene. Virus je pokazivao sposobnost hemadsorbcije, pripadao je serotipu 8 i genotipu II a u krvi, njegova koncentracija je bila 6.5 do 7.5 log HAU50/cm3. ASFV izolati koji su cirkulisali u centralnim regionima Ruske Federacije, imali su u svom genetskom materijalu inserciju od 10 parova baza u regionu I73R/I329L. Međutim, izolati ASFV koji su dobijeni iz regiona Irkutsk-a i Južne Osetije, kao i izolat Gruzija 2007/1 (FR682468.1), nisu posedovali ovu inserciju u genetskom materijalu.