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Comparative Analysis of Clinical and Biological Characteristics of African Swine Fever Virus Isolates from 2013 Year Russian Federation

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Authors' contributions

This work was carried out in collaboration between all authors. Authors NNV and IVS designed the study. Authors NNV, AAV, IVS, IYZ, SGR, VLG, OSP and AAS performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript and managed literature searches. Authors NNV, AAV, IVS, IYZ, SGR, VLG, OSP, AAS, NGZ and KNG managed the analyses of the study and literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Domestic pigs were inoculated with 50 (low) or 5000 (high) haemadsorbing doses (HAD_{50}) via intranasal (IN) or intramuscular (IM) routes, to investigate the pathogenesis of 5 Russian isolates of African Swine Fever Virus (ASFV) collected in 2013. Several clinical and virological parameters (including hemorrhagic syndrome, body fever and gross and microscopic lesions) were identical between the high and low dose groups for all 5 isolates, whereas duration of pyrexia, incubation

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period and clinical phase of infection differed between the high and low doses and between the 5 isolates. Additionally, pigs inoculated with high ASFV doses had the shortest mean survival time. A subsequent contact challenge experiment demonstrated that IN- and IM-inoculated pigs were able to transmit ASFV via direct contact with non-infected pigs.
Finally, our experiments indicated that these Russian ASFV isolates possessed variable pathogenicity. The isolates from wild boars had the lowest virulence and extended disease duration as compared to those from domestic pigs.

Keywords: African swine fever (ASF); challenge; ELISA; lymphocyte apoptosis; transmission; pathogenesis; domestic pig.

1. INTRODUCTION

African Swine Fever (ASF) is an economically important, highly contagious disease of pigs, ranging from highly lethal to subclinical forms. African swine fever virus (ASFV) is a single member of a new DNA virus family, Asfarviridae (A s f a r - African swine fever and related viruses) [1].

Virulent strains of ASFV can kill domestic pigs within about 5–14 days of infection with a mortality rate approaching 100%. Domestic pigs infected with less virulent isolates can survive infection and recovered pigs can gain immunity to subsequent challenge with low-virulent and related virulent viruses [2,3].

In nature ASFV can infect and replicate in wild boars, warthogs, bushpigs and soft ticks of the Ornithodoros sp., but a long term persistent infections were observed in warthogs and bushpigs [4]. The wild swine in Africa can stay infected over a long period without showing any symptom of the disease - and thus can be considered as natural reservoirs of the disease, in contrast to the European wild boar which appear to be highly susceptible [5].

Heterogeneous ASFV population contains some variants that have an advantage and supporting replication of the whole population in different host systems (susceptible animals or ticks). This complementation mechanism of ASFV population ensures their high survival in the wild nature [6].

ASFV is an enveloped virus with a large icosahedral capsid ~200nm in diameter, its capsid contains more than 50 proteins and consists of several concentric layers enclosing an electron-dense nucleoid, containing a double stranded DNA genome of approximately 190,000 bp [7,8].

Today more than 500 ASFV isolates and strains are classified into 22 genotypes based on p72-gene sequencing [9] with additional subtyping by p54 gene and CVR [10]. Alternative immune-serogroup based classification allows to distribute all ASFV strains between 10 groups including eight serogroup - based, one group of non-serotyped strains and one group consisting of heterogeneous isolates [11].

ASFV was introduced into the Republic of Georgia in 2007 and has subsequently spread to Russia, Armenia, Azerbaijan, Ukraine and European countries [12,13]. African swine fever was confirmed in the Republic of Belarus in 2013, and registered in Lithuania and Poland in 2014 [14].

In the Russian Federation, numbers of isolated outbreaks in conventional pigs were increasing in 2011 and 2012. It appears that backyard farms have been and are the main driving force for long-distance spread, as they are practicing animal feeding with kitchen waste without decontamination [15].

Outbreaks in the wildlife are likely a consequence of outbreaks in the backyard sector because the initial outbreaks occurred in the backyards exclusively, and then spread through the wildlife. Afterwards, the infected wild boars that acquired ASFV from the backyard farms were able to re-infect domestic swine forming a new “completely affected area” as shown on Fig. 1 [16,17].

However, the picture has changed significantly in 2012-2013: the affected ASF areas were formed in the wild - the virus started spreading from wild boar to boar, bypassing domestic pigs. Thus, there be two or more variants of the virus in the Russian Federation may, with different history of the long-distance spread [18].

Therefore, the goal of our research was to conduct a comparative analysis of the ASF virus isolates obtained from both domestic and wild animals.

2. MATERIALS AND METHODS

2.1 Virus and Cells

The new ASFV isolates: Boguchary 06/13 and Vyazma 08/13 from domestic pigs and Kashino 04/13, Karamzino 06/13 and K 08/13 from wild boars were isolated during outbreaks in the Russian Federation in 2013 (Table 1).

Initially, five ASF virus isolates were examined for their biological properties: haemadsorption, rate and timeframe of virus accumulation during three subsequent passages, in porcine alveolar macrophages (PAM) cell cultures. Samples were tested for infectious ASF virus presence using PCR and direct immunofluorescence assay. The passage duration was 7 days. Within the given period the microscopic examination was performed daily and the onset of haemadsorption and/or 65-70% CPE was recorded. If no virus accumulation was observed the blind passage was carried out as follows. Fresh PAM cells were inoculated with the virus-containing suspension of the previous passage at the ratio of 1:10.

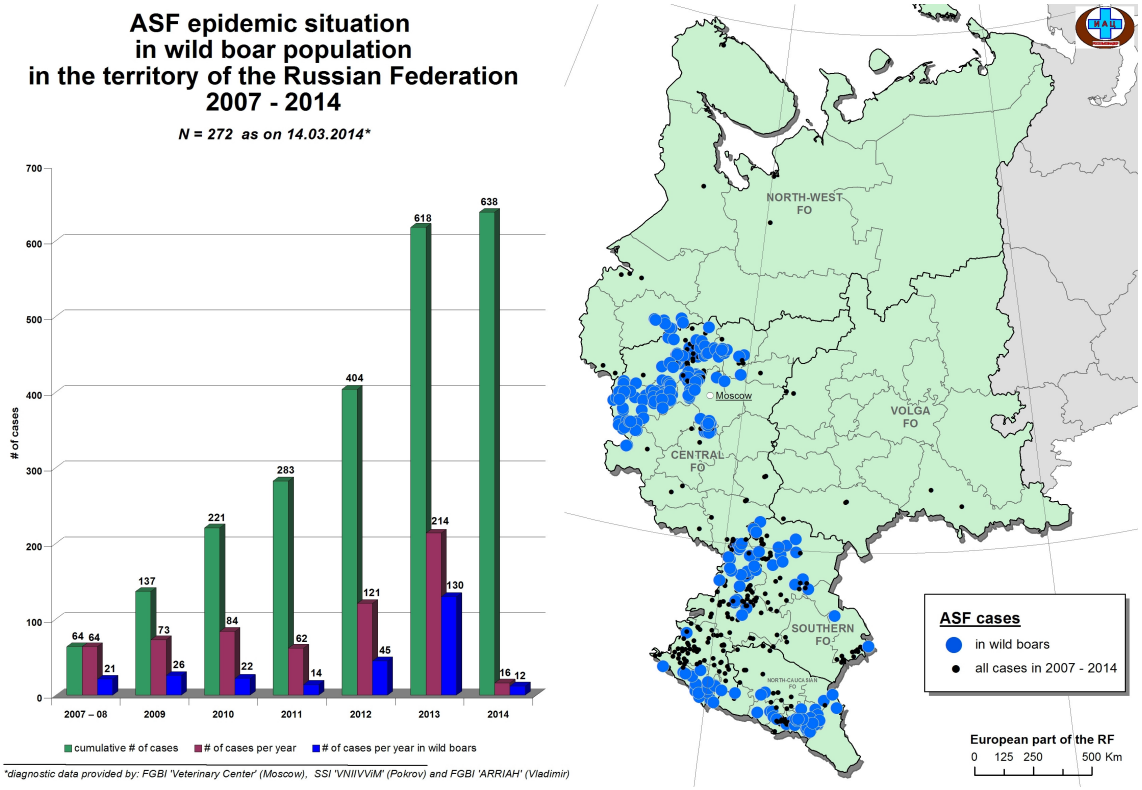

Fig. 1. ASF epidemic situation in wild boars in Russia

Table 1. Russian isolates 2013

Isolates and strain	Isolated from	Titer in PAM of 10% spleen suspension (lg HAD 50/cm^3)	Isolate keeps in collection of microorganisms of
Kashino 04/13	Wild boar	5.0	FGBI ARRIAH
Boguchary 06/13	Domestic pig	5.0	FGBI ARRIAH
Karamzino 06/13	Wild boar	4.5	FGBI ARRIAH
K 08/13	Wild boar	6.0	FGBI ARRIAH
Vyazma 08/13	Domestic pig	5.0	FGBI ARRIAH
Stavropol 01/08	Domestic pig	6.5	SRI NRIVVaM [19]

Virus titers in the clarified 10% suspension of the spleen from infected animals were determined using PAM and haemadsorbtion reaction. These 10% suspension supernatants were then stored as 10^{4-6} 50% haemadsorbing dose (HAD_{50}) aliquots at –70°C. The inoculum was prepared by initial 10-fold dilution of stock virus in Dulbeco's Modified Eagle's Medium (DMEM) (Gibco; Invitrogen, NY) containing 1% antibiotic (10,000 units/ml of penicillin and 10,000 g/ml of streptomycin) (Sintez Corporation, Russia) and subsequent additional dilution to achieve the desired viral dose in 2 ml of media.

The stock virus was passaged three times in primary PAM cell cultures derived from the lung alveolar as described [20]. The presence of the virus was confirmed by identification of infected cells by haemadsorption and titres expressed as doses calculated by the Reed-Muench method.

To assess the replicative properties following continuous cell lines CV-1, BGM, A4C2, PEK and PK (PEK and PK - pig embryo kidney; CV-1 and BGM - African green monkey kidney; A4C2 - hybrid PEK cells and splenocytes of pigs) were used.

2.2 Animals

Large white pigs from a herd confirmed to be free of viral disease agents were used for all the experiments. Pigs were approximately 2-3-month old and had 15–20 kg weight upon arrival. Animals were kept within the housing facilities for 7 days prior the onset of the experiment to allow them to acclimatize in the new environment.

This study was approved by the Institutional Animal Care and Use Committee (IACUC) of FGBI ARRIAH, and conducted in compliance with local and federal guidelines. For this particular study, we used the lowest numbers of pigs previously shown to permit detection of statistical significances among treatments considering welfare principles. This minimum number of pigs required per treatment group was verified by the statistician before starting the experiments.

2.3 Direct Inoculation Studies

Experiments were performed to compare two routes and two doses of inoculation for the 5 isolates. Four - six pigs were assigned to each of the 5 isolates inoculation route: intramuscular (IM) -2 (high or low dose), and intranasal (IN) - 4 (Table 2).

Inoculation route groupings were further subdivided into two inoculation dosage groups: low dose 5×10 HAD_{50} (n =2) and mid dose $5\times10^{3}HAD_{50}$ (n = 2-4). IM-inoculated pigs received a 2 ml injection of inoculum within the right cucullaris muscle. Pigs assigned to IN inoculation were placed in sternal recumbency and 2 ml of inoculum was instilled through the nares into the nasopharynx via a 10 cc disposable syringe (Medpolimer Company, Russia).

2.4 Direct Contact (DC) Transmission Studies

Additional experiments were performed to characterize ASFV transmission from IN-inoculated pigs and viral dynamics in pigs infected by direct contact. Infected (n = 4) and naïve pigs (n = 2) were initially housed in two separate rooms. Infected pigs were inoculated using the intranasal (IN) method as described above with 2 ml of inoculum at a dose of 5×10 HAD_{50} - $5\times10^{3}HAD_{50}$ of each isolate. Upon detection of pyrexia (rectal temperature higher than or equal to 40°C) in at least 2 infected pigs (by post inoculation day 6), contact pigs were transferred into the donor room and allowed to commingle with inoculated pigs. Contact animals were then observed for the onset and progression of clinical disease.

2.5 Clinical Evaluation and Sample Collection

For all studies, clinical evaluations were daily performed on all animals until death or termination of the experiment. The temperature and all clinical signs of each pig were recorded daily. The sample collection including whole blood with EDTA, clotted blood for the collection of serum, swab specimens from the nasal cavity and anus were performed on the day of inoculation at 0 days post inoculation (dpi) followed by sample collection through one day. Pigs were manually restrained for sample collection. Whole blood and serum were collected from the sine of cranial cava vein. Nasal and anal swabs were collected using sterile cotton swabs. Following collection, nasal and anal swabs were immediately immersed in 1 ml of DMEM with 5% antibiotic/antimycotic; then, all samples were transferred to cryovials and stored at –70°C until they were analyzed by virus isolation (VI) or PCR.

Table 2. The number of animals used in our experiments

Isolates and strain	Intranasal / low dose	Intranasal / high dose	Intramuscular / low dose	Intramuscular / high dose	Contact
Kashino 04/13	2	2	na	na	2
Boguchary 06/13	2	2	na	2	2
Karamzino 06/13	2	2	na	2	2
K 08/13	na	na	2	2	na
Vyazma 08/13	2	2	na	2	2
Stavropol 01/08	2	2	na	2	na

The post-mortem examination was performed following euthanasia (immediately) or natural death (within 4 hours). Tissues collected during post-mortem examination included: lung, thymus, liver, spleen, kidney, lymph nodes (retropharyngeal, submandibular, gastrohepatic, renal and inguinal). Urine was also taken by direct aspiration from the bladder (cystocentesis) at the time of necropsy. Collected tissue samples were placed in cryovials and stored at –70°C.

2.6 Immunofluorescence Assay

For the direct immunofluorescence assay anti-p72 monoclonal antibodies – FITC (Ingenasa, Spain) were used. For the inderect immunofluorescence assay cultures of PAM were either mock-infected or infected with ASFV, at a multiplicity of infection of 10.0 HAD_{50}/cell. Fluorescence observations were performed using Olympus fluorescence microscope (Japan) at 400 × magnification.

2.7 ELISA

Total IgG and IgM were determined as described by Reis A.L. [21]. After stopping the reaction with 0.1 M H_2SO_4 (50 µl per well) A450 was read on a PICON microplate Reader.

2.8 Statistical Analysis

The statistical analysis was carried out using repeated measures analysis of variance and STATGRAPHICS ® Centurion XV, version 1.15.02 software [22,23]. The statistical significance was considered as $p<0.05$. All data reported herein are not statistically significant unless explicitly stated.

3. RESULTS

3.1 Accumulation in Pam Cell Culture and Growth Kinetics of the Isolates

For all studied isolates we observed an increase in the virus titer from 4.5–5.0 to 5.0-7.5 lg HAD_{50}/cm^3 by the 3rd passage. Results of virus isolation of these 5 isolates in PAM cell culture are given in Table 3.

Replication parameters in PAM differed significantly for these 5 isolates. The haemadsorption activity in PAM cells infected with Boguchary 06/13, Vyazma 08/13 and K 08/13 isolates virtually remained unchanged during three passages whereas PAM cell infection with Kashino 04/13 and Karamzino 06/13 isolates resulted in low haemadsorption level characterized with a few of RBCs (10-30) attached to the infected cell during the 2nd-3rd passages.

The replication and accumulation rates of Boguchary 06/13, Vyazma 08/13 and K 08/13 isolates were 1-2 days faster and 0.5-1.0 lg HAD_{50}/cm^3higher, respectively, than those of Kashino 04/13 and Karamzino 06/13 isolates.

The results of the virus titration using haemadsorbtion reaction were confirmed by results of PCR [24] and direct immunofluorescence assay.

3.2 Testing of Isolates for their Properties in Continuous Cell Cultures

Three examined isolates yielding the highest virus load (K 08/13, Vyazma 08/13 and Karamzino 06/13) of 7.0 -7.5 lg HAD_{50}/cm^3 were selected for testing virus replication in continuous cell cultures. The following parameters were selected for the initial evaluation of replication: CPE development and morphology in CV-1, BGM, A4C2 and PK cell cultures, and compared haemadsorption presence and levels in PAM, time of haemadsorption progress during subpassaging in PAM, replication rate and virus accumulation level in tested cell cultures.

No virus replication was observed in CV-1 and BGM cell cultures during 5 passages since the decrease in the virus titers, from 6.0 to 2.0 lg HAD_{50}/cm^3, correlated with serial 10-fold dilutions of cultural materials.

Table 3. Properties of Russian isolates 2013 in PAM cell culture

Isolate	Passage number	Time of CPE occurrence (days)	Hemadsorption activity (RBC number per cell)	Virus accumulation titres (lg HAD $_{50}$/cm^3)	Period of virus accumulation (days)
Kashino 04/13	1	4-5	>50	6.23±0.37	8-10
	2	2-3	<50	6.84±o.45	6-7
	3	2-3	10-30	7.29±0.41	5-6
Boguchary 06/13	1	2-3	>50	7.45±0.12	6-7
	2	2-3	>50	7.52±0.02	4-5
	3	2-3	>50	7.43±0.34	4-5
K 08/13	1	1-2	<50	6.48±0.12	4-5
	2	1-2	<50	7.02±0.06	4
	3	1-2	<50	7.41±0.37	4
Vyazma 08/13	1	4	>50	5.97±0.13	5
	2	3	>50	6.89±0.31	4-5
	3	2	>50	8.03±0.12	4-5
Karamzino 06/13	1	4-5	10-30	4.35±0.17	7
	2	4	10-30	4.98 ±0.12	6-7
	3	3	10-30	5.25±0.22	6

The inoculation of A4C2, PEK and PK cell cultures yielded different results. The mean virus accumulation titre (lg HAD 50/cm^3) was 5.2 for A4C2, 5.5 for PEK and PK cells. CPE in cell cultures A4C2 and PEK was observed in the first passage for K 08/13 isolate, in the second and first - for Vyazma 08/13 isolate, in the second and third - for Karamzino 06/13 isolate, respectively. The presence of haemadsorption was detected by adding 0.01% RBC suspension to the cultural fluid. It should be noted that the non-specific RBC adsorption was observed on PK cell surface that was similar to that in intact cultural preparations.

A4C2 and PEK cell cultures appeared to be the most promising and were therefore selected for the further use as the virus replicated in those cultures producing haemadsorption, however, the periods of the virus accumulation at a given passage level were 9-12 days.

There were variations in haemadsorption levels on the A4C2 cell culture: the high haemadsorption levels were characteristic of K 08/13 and Vyazma 08/13 isolates, while haemadsorbtion of only 4-10 RBCs/cell were observed for Karamzino 06/13 (Fig. 2).

Thus, the 5 ASF virus isolates possessed variable biological characteristics *in vitro.*

3.3 Study of the ASFV Isolates Virulence in Pigs

A comparative study of biological properties of the five isolates collected in 2013 and one laboratory strain (Stavropol 01/08) was performed using conventional pigs [19].

Survival times of the pigs in four experimental groups, inoculated with different doses of the 5 ASFV isolates are presented on Fig. 3.

Post inoculation survival time was the shortest for the animals inoculated IM with high dose, with a minimum of 5 days for Stavropol 01/08 and maximum of 12 for K 08/13 isolate (Fig. 3). For intranasally inoculated with 5000 HAD pigs min survival time was 9 days for Vyazma 08/13 and for Stavropol 01/08 isolates, min survival time was 22 days for Karamzino 06/13 isolate. Fifty HAD dose inoculation resulted in min and max survival time of 8-9 days for Stavropol 01/08 and 22 days for Kashino 04/13 isolates. Commingle pigs survived for at least 14 (for Boguchary 06/13) and up to 22 days (Karamzino 06/13 and Kashino 04/13 isolates).

The levels and time frame of viremia of pigs inoculated with three of the tested isolates K 08/13, Kashino 04/13 and Boguchary 06/13 were determined by haemadsorption test and PCR.

The Kashino 04/13 isolate was characterized by low titers (1.0-2.0 lg HAD_{50}/cm^3) during the first days after challenge, whereas Boguchary 06/13 and K 08/13 isolates amplified to titers of 3.5-4.5 lg HAD_{50}/cm^3 by post-challenge day 3 (Table 4).

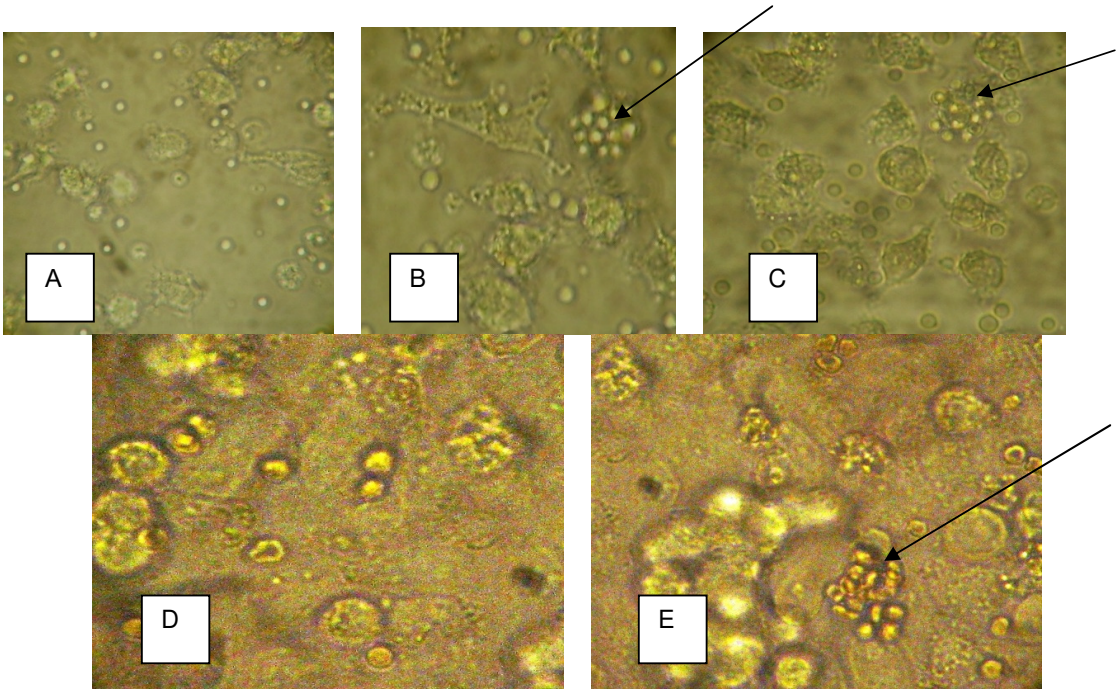

Fig. 2. Haemadsorption in porcine bone marrow cell culture A4C2

A –porcine bone marrow intact cell culture; B – high haemadsorption level in bone marrow cell culture from a pig inoculated with ASF virus Boguchary 06/13 isolate; C – low haemadsorption level in bone marrow cell culture from a pig inoculated with ASF virus Kashino 04/13 isolate; D – intact cell culture A4C2; E – high haemadsorption level in cell culture A4C2, inoculated with ASF virus Boguchary 06/13 isolate;
→ - ASFV-infected cells with attached RBCs

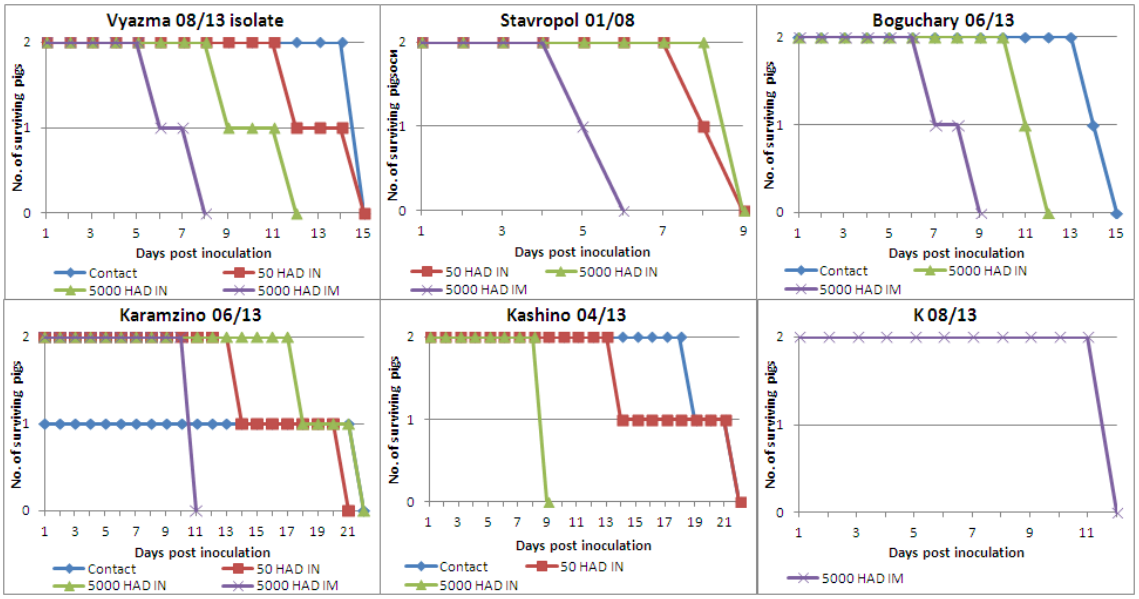

Fig. 3. Survival of pigs, inoculated with ASFV isolates

Table 4. Viremia levels in the animals inoculated with the ASFV isolates

Pigs inoculated with ASF virus		Days after challenge (virus titre is expressed as lg HAD_{50}/cm^3)									
		1	**3**	**5**	**7**	**9**	**11**	**13**	**15**	**17**	**19**
Kashino 04/13	at a dose of 5,000	1.5	3.0	5.0	7.5						
	HAD	1.0	2.5	5.0	7.5						
	at a dose of 50	0	1.0	3.0	6.5	4.0	5.0	6.0			
	HAD	0	1.5	3.5	6.5	5.0	6.5	5.5	6.5	7.5	7.0
	by contact	0	0	0	1.0	3.0	4.0	5.0	6.5	5.0	
		0	0	0	1.5	3.5	5.0	5.0	6.5	6.0	7.0
Boguchary 06/13	at a dose of 5,000	1.5	3.5	5.0	6.0	7.0	7.5				
	HAD	1.5	4.5	5.5	5.0	6.5					
	at a dose of 50	1.5	4.5	6.5							
	HAD	1.5	4.0	6.5	7.0						
	by contact	0	0	1.5	3.5	4.0	6.0	7.0			
		0	0	2.0	3.0	3.5	6.0	7.0			
K 08/13	at a dose of 5,000	1.5	4.5	5.5	6.5						
	HAD	1.5	3.5	4.5	5.0	6.5	7.0				
	at a dose of 50 HAD	1.5	4.0	5.5	5.0	7.0	6.5				
		1.5	2.5	5.0	5.0	6.5	6.0				

Pigs infected with Boguchary 06/13, Vyazma 08/13, Stavropol 01/08 isolates demonstrated hyperthermia in 3-4 days and resulted in death at 6-8 days post inoculation (dpi). Pigs inoculated IM with high dose Karamzino 06/13 isolate showed hyperthermia in 5-6 dpi and they died later – in 11 dpi. One out of four K 08/13 - infected pigs demonstrated rather rapid progression of clinical disease with body temperature increase (4 dpi.) and death on day 9. Other three pigs from the group developed clinical signs (with hyperthermia) only on 6-7 dpi. Animals were surviving for up to day 11 (day of the experiment termination).

The effect of the challenge route on disease progression in pigs was evaluated in a series of experiments. In one of the experiments, two pigs were inoculated intranasally with different ASFV isolates at a dose of 5,000 HAD per animal (Fig. 4).

The inoculated pigs developed clinical signs with hyperthermia on day 3-5 after inoculation by Kashino 04/13, Boguchary 06/13, Vyazma 08/13, Stavropol 01/08 isolates and died on day 9-12. The pigs inoculated intranasally with Karamzino 06/13 developed clinical disease with hyperthermia only on 13-15 dpi and died on 18-21 dpi.

Additionally, a subset of pigs was inoculated with a low dose (50 HAD) of virus-containing material via intranasal route.

The pigs inoculated with ASFV Stavropol 01/08 and Boguchary 06/13 developed clinical symptoms with body temperature increase by 4 dpi (Fig. 5) and subsequently died at 6-9 dpi. Similarly, pigs inoculated with Vyazma 08/13 developed clinical disease by 5-6 dpi, one of them died on 9 dpi, the other was euthanized on 14 dpi for collection of virus-containing blood. Pigs inoculated with Karamzino 06/13 and Kashino 04/13 developed hyperthermia between 9 and 15 dpi. One of the pigs inoculated with Kashino 04/13 was euthanized on 13 dpi for collection of virus-containing blood, the other pig died at 20 dpi. One Karamzino 06/13 isolate-inoculated pig died on 14 dpi, while the other pig from the group was slaughtered on 21 dpi (experiment termination date).

The post-mortem examination of carcasses of died and euthanized pigs from the aforementioned experiments demonstrated ASF-characteristic changes including splenomegaly, hyperplasia and hyperemia of regional lymph nodes, renal capsule hemorrhages, submucosal hemorrhages, etc. In some cases those changes were a little less pronounced (early dates of death) and in other cases they were more striking (later dates of death).

Using haemadsorption, direct immunofluorescence assay and PCR, ASFV (or its genome) presence was confirmed in blood and different organs (kidneys, lungs, spleen, etc.) of the euthanized/dead pigs.

3.4 Contact Challenge Study of the ASFV Isolates in Pigs

Observations in several contact challenge experiments for different ASFV isolates showed that disease progression coincided with terms of hyperthermia progression.

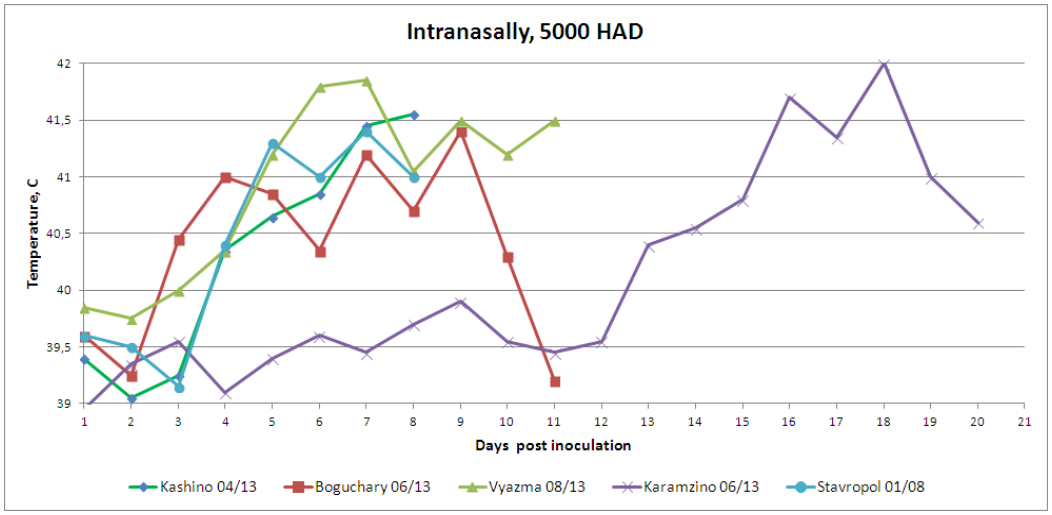

Fig. 4. Mean body temperature of the pigs inoculated intranasally with different ASFV isolates at a dose of 5,000 HAD

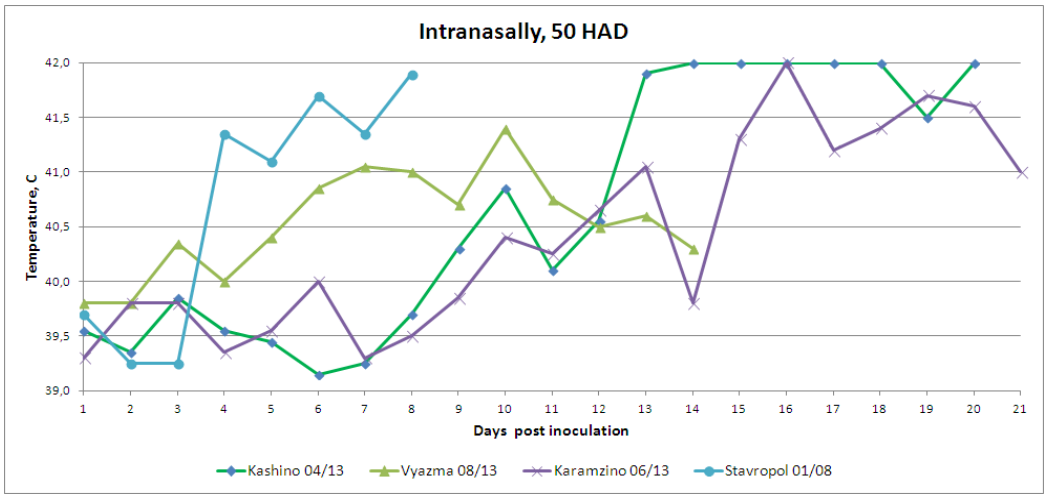

Fig. 5. Mean body temperature of the pigs inoculated intranasally with different ASFV isolates at a dose of 50 HAD (mean value)

In our experiments, commingle pigs demonstrated hyperthermia in 9-14 days, which was 6-8 days later as compared to hyperthermia onset in ASFV inoculated pigs. The Kashino 04/13 and Karamzino 06/13 commingle pigs survived for up to dpi 21, whereas the rest of the commingle pigs died or was euthanized (for the purpose of collection of virus-containing material) at day 14 and 19. The post-mortem examination of the dead and euthanized pigs demonstrated pathological changes characteristic of ASF. Direct immunofluorescence assay and PCR of different samples collected from those pigs confirmed the presence of ASFV.

Thus, all tested ASFV isolates were contagious for the pigs housed with ASFV inoculated animals.

3.5 Dynamics of the Antigen Accumulation and Antibody Production in the Pigs Inoculated with Different ASF Virus Isolates

The immunofluorescence assay demonstrated that the virus could be detected in blood samples from pigs inoculated with Kashino 04/13 isolate at a dose of 5,000 HAD on 1 dpi, at a dose of 50 HAD – on 2 dpi, and when the pigs were infected by contact the virus could be detected on day 5

post contact. The virus could be detected in blood from pigs infected with Boguchary 06/13 isolate, at a dose of 50 HAD and 5,000 HAD a day post inoculation and from pigs infected via contact – on 4 dpi. In the animals infected with the K 08/13 isolate the level of the virus accumulation in blood significantly varied that subsequently affected the time of animal death.

Results of virus isolation were confirmed by testing blood smears for the presence of ASFV by direct immunofluorescence assay.

On 3 dpi 3-7 fluorescent cells were detected by direct immunofluorescence assay in the prepared blood smears and nasal swabs from all pigs infected at a dose of 5,000 HAD.

In 5 and 7 days post inoculation specifically fluorescent cells (20-100) were detected in all smears prepared from blood and organ samples of pigs infected with Kashino 04/13 isolate at a dose of 5,000 HAD and Boguchary 06/13 isolate at a dose of 50 HAD with the highest number of cells detected in smears of submandibular lymph nodes, spleen and lungs.

Serum samples collected during the experiment were also tested for the presence of early IgM by indirect haemagglutination assay and IgG – by competitive ELISA.

Early antibody titers also varied in animals infected at a lower dose of 50 HAD. A delayed increase of antibody titers was observed in animals inoculated with the Kashino 04/13 isolate; on day 5 the antibody titers only reached 1:16-1:32, while the serum antibody titer against Boguchary 06/13 on day 5 was >1:512 in the surviving pig.

Results of the test for early IgM using monoclonal antibodies to porcine immunoglobulin M correlated with the indirect haemagglutination data excluding the findings for samples collected one day post challenge in which the antibody titer was no more than 1:8.

The study of the dynamics of antibody production by competitive ELISA showed that IgG antibodies were detectable only on day 13-19.

The study of nasal and anal swabs from infected animals demonstrated that the ASF virus genome could be detected by PCR on 5 dpi in all infected pigs (data not shown).

Apart from the virus detected by PCR and direct immunofluorescence assay, specific antibodies to the ASF virus were detected by indirect immunofluorescence assay in samples of serum, spleen and submandibular lymph nodes collected from the animals died on day 5-7 and later post inoculation (data not shown).

4. DISCUSSION

Currently one of the key questions is the homogeneity of the ASFV pool circulating in Russia. Highly virulent ASF virus was initially introduced into Russia. It caused the acute form of the disease in pigs, with typical hemorrhagic syndrome and multi system organ lesions, high and stable levels of viremia, absence of specific antibodies in sera and 100% mortality rate [25].

We hypothesize that 6 years after ASFV was introduced to Russia, the virus could acquire new distinct biological properties. For example, the endemic state of latent ASF in the wild boar population in Portugal was formed in 5-7 years after introduction of the virus into the country. Additionally, the presence of attenuated variants of ASF virus was recorded in ticks [26].

However, currently there is no evidence that ticks serve as vectors of the ASFV in Russia. A decrease in the virus virulence after its long-term persistence in the wild with high density of affected animals is a common result of the virus adaptation to a host to improve fitness. The presence of the animals with decreased sensitivity to a pathogen that serve as the only natural reservoir allows the pathogen to establish long-term persistence in the wild [27].

The biological properties were best described for the initial isolate Georgia 2007/01 and the strain Stavropol 01/08. [28,19] It was demonstrated, that the Caucasian isolates are highly virulent for domestic pigs and wild boars of all age groups. No apparent differences were found in the disease course in domestic pigs and wild boars. However, a moderate contact infection was recorded that was caused by a direct contact with infected blood.

In this study, we chose to investigate the changes, accumulated by ASV virus over 6 years compared to isolate Stavropol 01/08. The most promising isolates for the study were the ones with unknown number of passages in the wild, as the epizootic analysis indicated that the isolates with an anthropurgic spread vector normally undergo only a few passages in different hosts.

Belyanin et al. [25] stated that the ASF virus circulating in Russia has not changed significantly: incubation period during the experimental challenge on pigs was 4,0 (3,0-5,0) dpi, the duration of the disease - 6,5 (5,0-8,0) dpi, virus-specific pathological changes were observed in all organs (liver, kidneys, spleen, lungs and lymph nodes), such as haemorrhagic syndrome, constantly high viremia levels, absence of specific antibodies and 100% mortality rate.

The haemadsorption levels, as well as the period and the level of Kashino 04/13 and Karamzino 06/13 virus accumulation were lower as compared with isolates Boguchary 06/13, Vyazma 08/13 and K 08/13. Based on Table 3 analysis, Karamzino 06/13 had a substantial replication defect in swine macrophages, which might have contributed to the attenuation observed *in vivo*.

Three isolates K 08/13, Vyazma 08/13 and Karamzino 06/13 with the highest haemadsorption level in primary cell culture of porcine alveolar macrophages were selected for cultivation in continuous cell cultures (CV- 1, BGM, A4C2 and PK) for 5 consecutive passages. According to our results, continuous cell lines A4C2 and PK were the most suitable for cultivation, accumulation and measurement of the haemadsorption level of ASFV isolates.

The clinical picture for the animals infected with high doses (5000 HAD) varied between the isolates. For the Kashino 04/13, Boguchary 06/13, Vyazma 08/13, Stavropol 01/08 intranasally inoculated animals, hyperthermia onset was observed at 3-5 dpi and the animals were dead on 9-12 dpi However, for the pigs intranasally inoculated with isolate Karamzino 06/13 hyperthermia developed 9-10 days later. Thus, the ASFV isolates from wild boars (Karamzino 06/13 and Kashino 04/13) induced variable clinical symptoms (hypethermia onset, disease progression and survival time) in the experimental animals infected with high dose. However, all the animals infected with the isolates from domestic pigs (Boguchary 06/13, Vyazma 08/13, Stavropol 01/08) developed clinical symptoms and/or died within the same timeframe.

Animals, inoculated with the low dose (50 HAD) of ASFV isolates from domestic pigs developed hyperthermia on 4-5 dpi, and died at 6-9 dpi However, animals infected with the isolates from wild boars developed hyperthermia between 9-15 dpi, and died at 14-21 dpi It suggests, that clinical picture and disease prognosis associated with the low dose allows to discriminate between the isolates from domestic pigs and wild boars. Additionally, it demonstrates that the isolates from wild boars in this study possess biological characteristics distinct from those described by Belyanin S.A. et al. [29]. According to the authors, it indicates the pathogenicity of the Russian virus isolates, which may only cause severe and acute forms of the disease [29].

Analysis of the impact of infectious dose quantities on the severity of the disease and the lifespan of animals showed no substantial differences between the high and low doses. Pigs, infected with ASF virus isolates Boguchary 06/13, Vyazma 08/13, Stavropol 01/08 in high doses (5000 HAD) died 1-2 days earlier or at the same time as pigs infected in low doses (50 HAD).

Hereby, our experiments indicated that in case of inoculation of pigs by the virus isolated from wild boars (Karamzino 06/13 and Kashino 04/13 isolates), periods of the disease progression and duration were more prolonged compared to the other isolates examined. The form of the disease may be characterized as subacute.

The results of Kashino 04/13 isolate genome pyrosequencing (unpublished; GenBank Data Libraries under Accession No. KJ747406) confirmed the presence of changes in its DNA compared to the genome of isolate Georgia 2007/01.

Identified clinical and biological differences in Kashino 04/13 and Karamzino 06/13 isolates from wild boars suggest about circulation of virus modified forms in nature. However, further investigations on correlation between molecular and biological properties of the virus are required.

Thus, it can be assumed, that infection with low dose in the field (especially for old animals), may result in an effect, characteristic for moderate virulent strains, which leads to development of virus carriers / or the appearance of chronically infected animals.

Successful confirmation of this hypothesis may be crucial for the development of strategies for the ASF eradication program to prevent ASFV endemic.

5. CONCLUSION

The goal of this research was to conduct comparative analysis of Russian Federation 2013 ASFV isolates obtained from both domestic swine and wild boar in order to understand if the differences observed in the epizootological patterns (boar to boar and swine to swine transmission) could be related with the differences in studied isolates. While the genetic basis of virus-host interaction is a very interesting question, the further investigations are required to support the conclusions drawn. While there might be differences in virulence (delayed onset of clinical symptoms, modest and transient reductions in viremia, and increased time to death) for wild boar isolates the data are convincing.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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