UDC 619:616.98:579.852.11

DOI 10.29326/2304-196X-2018-4-27-21-25

ANALYSIS OF CHANGES IN AFRICAN SWINE FEVER VIRUS GENETIC STRUCTURE AND BIOLOGICAL PROPERTIES DURING ADAPTATION TO CONTINUOUS CELL CULTURE

Ali Mazloum[1], N. G. Zinyakov[2], A. S. Pershin[3], I. V. Shevchenko[4], I. Yu. Zhukov[5], D. N. Fedoseyeva[6], D. V. Sharypova[7], A. S. Igolkin[8], N. N. Vlasova[9]

[1] Specialist of the Reference Laboratory for African swine fever, FGBI "ARRIAH", Vladimir, Russia, *e-mail: ali.mazloum6@gmail.com*

[2] Senior Researcher, Candidate of Science (Biology), FGBI "ARRIAH", Vladimir, Russia, *e-mail: zinyakov@arriah.ru*

[3] Researcher, Candidate of Science (Veterinary Medicine), FGBI "ARRIAH", Vladimir, Russia, *e-mail: pershin_as@arriah.ru*

[4] Researcher, Candidate of Science (Biology), FGBI "ARRIAH", Vladimir, Russia, *e-mail: shevchenko@arriah.ru*

[5] Leading Biologist, FGBI "ARRIAH", Vladimir, Russia, *e-mail: zhukov@arriah.ru*

[6] Specialist of the Reference Laboratory for African swine fever, FGBI "ARRIAH", Vladimir, Russia, *e-mail: fedoseeva@arriah.ru*

[7] Specialist of the Reference Laboratory for African swine fever, FGBI "ARRIAH", Vladimir, Russia, *e-mail: sharipova@arriah.ru*

[8] Head of Laboratory, Candidate of Science (Veterinary Medicine), FGBI "ARRIAH", Vladimir, Russia, *e-mail: igolkin_as@arriah.ru*

[9] Chief Researcher, Doctor of Science (Biology) FGBI "ARRIAH", Vladimir, Russia, *e-mail: vlasova_nn@arriah.ru*

SUMMARY

Functions of many African swine fever virus genes and multigene family members have not been yet understood. In particular, no virus genes directly associated with the virus virulence in pigs have been identified. Identification of such genes will enable preparation of deletion mutant ASF virus strains as well as development and testing pilot safe vaccines based on the said virus strains. Comparative analysis of the virus biological characteristics and detection of differences in its genome structure affecting certain phenotypic features is the main method used for the virus basic pathogenicity and immunogenicity examination. The most interesting and effective approach to addressing this problem is analysis of changes in the gene structure during ASF virus adaptation to replication in continuous cell culture. The said factors have made continuous cell culture-adapted variant ASF virus preparation necessary. Variant viruses with modified biological features were prepared during adaptation of ASFV Odintsovo 02/14 isolate to replication in CV-1 cell culture. Lethality level was 16.7% when pigs were infected with adapted variant virus at 30th passage and survived animals became resistant to reinfection with homologous virulent ASFV Arm 07 isolate. It should be noted that the virus passage in non-permissive cell culture up to 30 serial passages did not result in enormous changes in its genotype; however, a large 3,000 bp deletion similar to that one in continuous Vero-cell culture-adapted BA71V strain genome appeared in the right terminal variable region of the genome.

Key words: African swine fever virus, adaptation, continuous CV-1 cell culture, genotyping, bioassay, whole genome sequencing, genetic structure, biological properties.

INTRODUCTION

African swine fever (ASF) – viral disease of both domestic and wild animals characterized by high mortality rate as well as contagiousness and having hyperacute, acute, subacute, chronic and unapparent forms. As a rule at the beginning of the epidemic the virus causes a hyperacute and acute form of the disease with a high pathogenicity level (mortality approaching 100%), then virulence of the circulating virus gradually decreases [4–6].

Thus, an ASF-associated outbreak was reported in 1960 in Portugal and it caused 100% mortality in infected animals and in eight years of the virus circulation a low-virulent isolate was detected the administration of which induced immunity to the consequent infection with the original isolate [12].

ASF spread causes great economic losses comprised of direct and indirect expenditures on veterinary and sanitary measures as well as quarantine measures, stamping out of the entire animal population in the outbreak and in the infected zone as well as restrictions in the international trade [4, 8].

It is obvious that for ASF outbreak containment and eradication it is necessary to rapidly detect diseased animals as early diagnosis is most effective for animal health protection and is a quite complicated component of any efficient disease surveillance system.

One of the basic stages in the disease spread prevention is the epizootic chain rupture, i. e. blocking agent transmission routes and elimination of the disease spread

factors. For this purpose the disease spread routes and factors should be determined during performance of surveillance measures.

The unique properties of the virus together with the unavailability of agents for disease specific prevention and treatment make ASF a global threat to pig production in our country and in the world. That's why the development and improvement of efficient ASF control measures demand further comprehensive studies of the disease and its agent.

ASF virus is the only DNA-containing arbovirus. It's size varies from 170 to 193 kbp and contains 150–167 open reading frames (ORF), depending on the isolate. Open reading frames are located close to each other, have overlapping regions and are read from both DNA chains [10, 11].

Functions of many genes and members of multigene ASFV families have not been fully understood. In particular, genes responsible for the virulence of the virus have not been determined yet. Identification of these genes will make creation of the ASFV deletion mutant strains as well as development and testing of experimental safe vaccines on their basis possible [6, 10].

The basic method for studying the ASFV pathogenicity and potency is comparative analysis of the virus biological properties and detection of differences in the structure of its genome influencing certain phenotypic characteristics.

The most interesting and effective approach to solve the problem is analysis of the gene structural changes during ASFV adaptation to reproduction in the continuous cell line which allows to find out the nature of pathogenicity of the studied variant viruses.

These factors led to the necessity of preparing the ASF variant virus adapted to growth in the continuous cell line. The research was aimed at analysis of changes during the process of ASFV isolate (Odintsovo 02/14) adaptation.

MATERIALS AND METHODS

The ASFV-containing samples were obtained by the FGBI "ARRIAH" specialists during the process of virus adaptation in the CV-1 cell culture.

CV-1 primary and continuous cell culture was grown in Eagle's medium adding 10% bovine fetal serum.

Bioassay test was performed for the purpose of studying biological properties of the adapted variant virus, determining its capacity to cause the disease and investigating possible changes of the agent's properties by inoculating piglets with the material under investigation according to "Methodical recommendations for biological assay performance on pigs infected with ASFV" (FGBI "ARRIAH").

At the same time contagiousness level in the tested virus samples was assessed. For this purpose healthy and weak pigs were used in the experiment. The animals were observed on a daily basis with performance of visual examination and measuring rectal temperature of each piglet according to the "Methodical recommendations for assessment of clinical signs and post-mortem lesions in case of experimental infection with the African swine fever virus (FGBI "ARRIAH")". Blood samples as well as nasal and rectal swabs were collected from all animals once each three days for detection of systemic infection allowing virus isolation.

Hemadsorption test performed in swine bone marrow cell culture, PCR and DIFA tests were all used for detection of ASFV in the tested samples and to determine viremia level according to "Methodical recommendations for preparation of pig type-specific serum against African swine fever virus for performing hemadsorption inhibition test", "Methodical recommendations for ASFV genome detection by RT PCR" and "Methodical recommendations for ASFV detection in blood samples and pathological material collected from dead and emergency killed pigs using direct immunofluorescence assay (DIFA)" (FGBI "ARRIAH").

For PCR performance "Test system for ASFV diagnosis by the real time PCR (FGBI "ARRIAH") was used.

To analyze changes in the genome during the process of adaptation, whole genome sequencing was performed using Nextera XT DNA Library Prep Kit reagents and Illumina MiSeq sequencer (Illumina, USA).

Table 1
ASFV reproduction (Odintsovo 02/14 isolate) during adaptation to growth in the CV-I cell culture *(n = 5)*

Passage number	Virus titer (lg $HADU_{50}/cm^3$)	Accumulation period (days)
1	5.12 ± 0.25	14
5	4.65 ± 0.32	12
10	5.46 ± 0.21	9–10
15	6.09 ± 0.24	8–9
20	6.21 ± 0.17	7–8
25	7.08 ± 0.19	7
30	7.31 ± 0.12	7

RESULTS AND DISCUSSION

To assess the possibility of detecting changes in ASFV biological properties and attenuation it is necessary to perform adaptation to growth in the continuous cell culture and determine biological, molecular and genetic characteristics of the virus.

Adaptation. The primary virus-containing material of Odintsovo 02/14 isolate was accumulated in the swine bone marrow cell culture with the titer of 7.21 ± 0.36 lg $HADU_{50}/cm^3$. The prepared virus-containing material, 1:10 (v/v), was inoculated in the CV-1 cell culture where further on 30 subsequent passages were performed using the same method. Herewith, it should be noted that effective ASFV reproduction in CV-1 cell culture was registered only starting from passage 12. The results of the experiments are given in Table 1.

During passaging in the CV-1cell culture at passage 20 (Table 1) the virus titer was 6.21 ± 0.17 lg $HADU_{50}/cm^3$, and the accumulation period declined from 14 days to 7–8 days.

When assessing the changes in hemadsorbtion process it was observed that the number of red blood cells attached to the infected cell of the adapted virus of passages 15–29 decreased (from 40–50 to 30–40) and at passage 30 this parameter decreased to 20–30.

Biological assay. When testing infectivity of the prepared ASFV strain ASF/ARRIAH/CV-1 six piglets were inoculated with a virus-containing suspension intramuscularly at a dose of 10 HADU/animals, two more piglets were kept together with the infected piglets for the as contact animals. As a result of the intramuscular and contact infections with apparent clinical signs three out of eight piglets

died, i.e. general mortality for ASF/ARRIAH/CV-1 strain was 37.5%.

The performed tests revealed that the virulence of the ASF/ARRIAH/CV-1 strain as well as its capability to induce post-infection virus-specific antibodies detected in blood serum, diluted up to 1:2000 within a period of up to 14 days post infection, using a commercial ELISA diagnostic kit, decreased.

ASFV adapted to CV-1 continuous cell culture from the 30th passage (ASF/ARRIAH/CV-1/30) was also used to perform bioassay test on piglets using a dose of 10 HADU/animal, herewith, the experimental animals were divided into three groups: two piglets – contact animals (group 1), two piglets – animals weakened by bacterial infection (group 2) and four piglets – normal animals (group 3). Body temperature was measured in all animals. Thermography results are demonstrated in Figure 1.

The results given in Figure 1 demonstrate that the body temperature of piglets from the control group did not raise higher than 40.5 °C during the entire observation (28 days), which is indicative of no contact virus transmission.

Animals weakened by bacterial infection demonstrated fever starting from day 5, on day 11 one piglet died. During its autopsy ASF-associated lesions were detected – hyperemia and hypertrophy of lymphatic nodes, enlarged spleen, and lung inflammatory lesions.

Group 3 animals demonstrated short-term temperature rise up to 41–41.3 °C on day 7–9. All animals of this group remained alive until the end of the observation period (Table 2).

So, mortality of animals from groups 2 and 3 was 16.7 % , significant change in biological properties of the adapted variant ASF virus from passage 30 (decrease in mortality from 85.7% to 16.7%) is indicative of probable changes in its genome.

Challenge test. During the bioassay test, when pigs were infected with the adapted ASFV from passage 30, five out of six pigs (groups 2–3) and two contact pigs (group 1) survived. To study the immune status and to determine whether the animals are resistant to ASFV a challenge test was performed. Reference ASFV isolate Arm 07 was administered to pigs intramuscularly at a dose of 1000 HADU/animals. The survived contact animals from Group 1 were used as control animals. Body temperature of all experimental piglets was measured daily during the entire observation period (30 days post the challenge test).

On days 6–7 control piglets died demonstrating ASF signs. After the challenge test four out of five piglets that survived after the first inoculation demonstrated indolent conjunctivitis within the specified period and their temperature remained normal; piglet 5 (from the group of weakened animals) demonstrated swollen joints and bleeding as well as necrotic lesions on skin, characteristic of ASF.

As all the animals survived the challenge test and no other temperature rises or ASF clinical signs were observed, they have obviously developed protective immunity.

Genome analysis. Sequencing of C-terminal region of B646L gene, coding the major capsid protein p72, the adapted variant ASVF virus from passage 30 was referred as genotype II. Consequently, changes observed during the virus adaptation to growth in the nonpermissive cell culture did not affect the region responsible for its classification characteristics.

Whole genome sequencing of the viral DNA extracted from CV-1 cells was performed according to the manual to determine the causes of decrease in the adapted virus virulence and carry out comparative analysis of the nucleotide sequence [1].

The comparative analysis of the whole genome sequence of the adapted variant ASFV (ASF/ARRIAH/CV-1 strain, passage 30) and Georgia 2007/1 isolate revealed the presence of point mutations in four genes (M1249L, NP419L, E199L and I196L). According to L. K. Dixon et al. their function is determined in the following order: NP419L – DNA-ligase, is involved in the virus replication; M1249L – is involved in the virus replication, exact function is unknown; E199L – structural protein, being a transmembrane domain of J-18L protein and I196L – function is not known [3].

Besides, as it has been previously found out a large deletion, covering a p22-encoding gene and genes of multigene families MGF100 and MGF360 in the right variable terminal region of the genome were observed in the adapted BA71V strain [9]. Analysis of the ASF/ARRIAH/CV-1 genome sequence also detected a large deletion (2,945 bps) in the region 180 975–183 920 which resulted in the loss of genes L7L, L8L, L9L, L10L, L11L and MGF360-18R.

According to L. K. Dixon et al. L10L belongs to p22 gene family, L7L, L8L, L9L and L11L functions have not been identified yet and gene MGF360-18R belongs to family MGF360, responsible for ASFV virulence [3].

So, it can be assumed that there is a correlation between the changes in the right variable terminal region of the genome covering genes L7L, L8L, L9L, L10L, L11L and MGF360-18R, and biological properties of the virus. This is confirmed by experimental tests performed on animals and correlates with results obtained by other researchers in Spain, Great Britain and the USA [2, 3, 7, 9].

CONCLUSION

As a result of the tests performed ASFV strain (ASF/ARRIAH/CV-1) from passage 20 adapted to growth in the CV-I continuous cell culture was prepared and deposited in the FGBI "ARRIAH" microorganism strain collection. The strain was characterized according to basic biological properties, the possibility of its use for further adaptation was determined, and patent application was submitted.

During the process of the ASFV Odintsovo 02/14 isolate adaptation to reproduction in the CV-1 cell culture the

Fig. 1. Thermography, infected animals

1.1 and 1.2 (k) – animals from Group 1;
2.1 and 2.2 (o) – animals from Group 2;
3.1, 3.2, 3.3 and 3.4 – animals from Group 3/

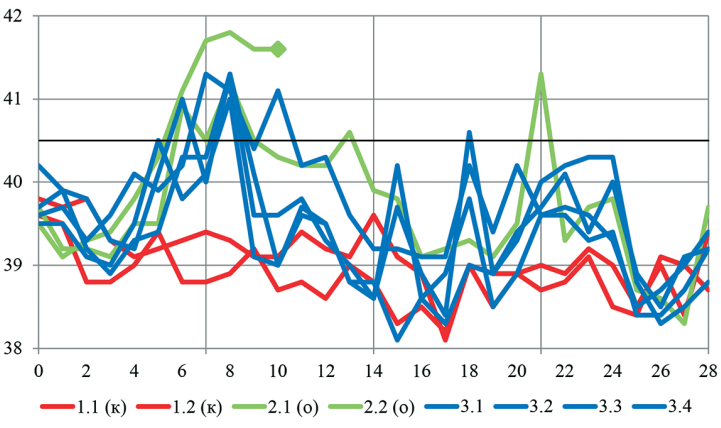

Table 2
Temperature parameters in experimental animals on day 5–14 post infection with the 30 passage ASFV, adapted to CV-1 cell culture

Group number	Animal number	Days post infection							
		5	7	8	9	10	11	12	14
1	1.1 (к)	39.4	38.8	38.9	39.2	38.7	38.8	38.6	39.0
	1.2 (к)	39.2	39.4	39.3	39.1	39.1	39.4	39.2	39.1
2	2.1 (о)	**40.5**	40.5	41.2	40.5	40.3	40.2	40.2	40.6
	2.2 (о)	**40.3**	41.7	41.8	41.6	41.6	†	†	†
3	3.1	39.4	40.0	41.3	39.6	39.6	39.8	39.3	39.0
	3.2	39.9	40.1	41.0	39.1	39.0	39.7	39.5	38.8
	3.3	39.9	40.3	41.3	40.1	39.0	39.6	39.5	38.8
	3.4	39.5	40.3	41.1	40.4	40.1	40.2	40.3	39.6

† – Animal death.

variants of the virus with modified biological properties were prepared. As during the biological assay test using the variant virus from passage 30 contact pigs remained intact and nonimmune it can be assumed that this variant virus has lost its contagiousness characteristic of the primary isolate Odintsovo 02/14 [6].

When the pigs were inoculated with the adapted variant virus from passage 30 mortality was 16.7%, and all the survived pigs acquired resistance to the repeated inoculation with the virulent isolate Arm 07.

It should be noted that the virus passaging in the non-permissive cell culture for up to 30 subsequent passages did not result in the genotype changes, a large deletion of 3000 bps similar with the one on the BA71V strain genome adapted to growth in the Vero continuous cell culture appeared in the right variable terminal region of the genome.

REFERENCES

1. Glick B. R., Pasternak J. J. Molecular Biology. Principles and Application: translated from English. M.: Mir, 2002 (in Russian).

2. African swine fever virus Georgia isolate harboring deletions of MGF360 and MGF505 genes is attenuated an swine and confers protection against challenge with virulent parental virus. V. O'Donnell, L. G. Holinka, D. P. Gladue [et al.]. *J. Virol.* 2015; 89 (11): 6048–6056; DOI: 10.1128/JVI.00554-15.

3. African swine fever virus replication and genomics. L. K. Dixon, D. A. Chapman, C. L. Nethertona, C. Upton. *Virus Res.* 2013; 173 (1): 3–14; DOI: 10.1016/j.virusres.2012.10.020.

4. African swine fever. *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. OIE*. 7th ed. Paris, 2012; 2, Chap. 2.8.1: 1067–1081.

5. African swine fever. Technical Disease Card. OIE. URL: http://www.oie.int/fileadmin/Home/eng/Animal_Health_in_the_World/docs/pdf/Disease_cards/AFRICAN_SWINE_FEVER.pdf (access date: 18.09.18).

6. Biological properties of African swine fever virus Odintsovo 02/14 isolate and its genome analysis. A. A. Elsukova, I. V. Shevchenko, A. A. Varentsova [et al.]. *Int. J. Environ. Agric. Res.* 2017; 3 (10): 26–37.

7. Deletion of African swine fever virus interferon inhibitors from the genome of a virulent isolate reduces virulence in domestic pigs and induces a protective response. A. L. Reis, C. C. Abrams, L. C. Goatley [et al.]. *Vaccine.* 2016; 34 (39): 4698–4705; DOI: 10.1016/j.vaccine.2016.08.011.

8. Detection of African swine fever antibodies in experimental and field samples from the Russian Federation: Implications for control. L. Mur, A. Igolkin, A. Varentsova [et al.]. *Transbound. Emerg. Dis.* 2016; 63 (5): e436–440; DOI: 10.1111/tbed.12304.

9. Genome sequence of African swine fever virus BA71, the virulent parental strain of the nonpathogenic and tissue-culture adapted BA71V . J. M. Rodríguez, L. T. Moreno, A. Alejo [et al.]. *PLoS ONE*. 2015; 10 (11):e0142889; DOI: 10.1371/journal.pone.0142889.

10. Genomic analysis of highly virulent Georgia 2007/1 isolate of African swine fever virus. D. A. Chapman, A. C. Darby, M. Da Silva [et al.]. *Emerg. Infect. Dis.* 2011; 17 (4): 599–605; DOI: 10.3201/eid1704.101283.

11. Genotyping field strains of African swine fever virus by partial p72 gene characterization. A. D. Bastos, M. L. Penrith, C. Crucière [et al.]. *Arch. Virol.* 2003; 148 (4): 693–706; DOI: 10.1007/s00705-002-0946-8.

12. Related strains of African swine fever virus with different virulence: genome comparison and analysis. R. Portugal, J. Coelho, D. Höper [et al.]. *J. Gen. Virol.* 2015; 96 (Pt 2): 408–419; DOI: 10.1099/vir.0.070508-0.

Submitted on 30.11.18
Approved for publication on 06.12.18