

DETERMINATION OF THE 1L-5-6L MGF110 GENES INFLUENCE ON THE BIOLOGICAL PROPERTIES OF THE AFRICAN SWINE FEVER VIRUS (*ASFARVIRIDAE*; *ASFIVIRUS*) “VOLGOGRAD/14C” *IN VIVO*

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(Received 22 February, Accepted 07 June 2024)

African swine fever continues to pose a global agricultural problem due to the absence of vaccine prevention and the high cost of anti-epizootic measures. This study examines the functional role of the African swine fever virus (ASFV) 1L-5-6L multigenic family 110 genes *in vivo*. Four clinically healthy Large White pigs were used in this study. Two groups of animals were inoculated with either the parental strain or the deletion variant, respectively. For subsequent challenge infection, the homologous virulent strain “Stavropol 01/08” was used. Blood samples were collected at specific time intervals. The ASFV infectious activity was determined by titration in porcine blood-derived macrophages. Virus-specific antibodies to the ASFV p30 protein were detected using an enzyme-linked immunosorbent assay (ELISA).

The results of real-time polymerase chain reaction (PCR) showed a significant difference in Ct values between samples from the two groups of animals. The determination of ASFV infectious activity in blood samples demonstrated the presence of the virus in animals immunized with the parental strain. The virus was not detected in samples from animals immunized with the deletion strain. The ELISA method demonstrated the presence of p30 protein antibodies in serum samples from 10 to 14 days after immunization with the parental strain, while no antibodies were detected in serum samples from animals immunized with the deletion strain.

The properties of the ASFV recombinant strain “Volgograd/D(1L-5-6L) MGF110” were studied in an *in vivo* experiment. It was found that the deletion strain does not reproduce in animals, unlike the parental strain.

Keywords: African swine fever virus, multigenic family 110 (MGF110), PCR, ELISA.

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INTRODUCTION

African swine fever (ASF) is a contagious viral disease of domestic pigs and wild boars characterized by hemorrhagic fever and mortality rates up to 100% [1]. Currently, there are no specific methods for the prevention of this disease. The most promising approach is considered to be the creation of artificially attenuated ASF virus strains through the deletion of one or more genes [2,3]. Despite many years of research by scientists worldwide, a safe and effective vaccine that fully protects the population from infection has not yet been developed. Therefore, further studies of the functions of individual ASF virus genes is necessary. The terminal regions of the virus genome, which mainly consist of multigene families containing several genes, are the most variable genome regions [4,5]. However, the functions of individual genes within these multigene families have not been fully elucidated.

The aim of this study was to investigate the functional role of the MGF110 1L-5-6L genes in viral replication and their influence on the host immune response during African swine fever in an experiment on susceptible animals.

MATERIALS AND METHODS

Viruses

The parental strain "Vologograd/14c" and its recombinant deletion variant "Vologograd/D(1L-5-6L) MGF110" (genotype II) were used to infect animals.

Pigs were inoculated intramuscularly with a dose of $10^{3,0}$ HAU₅₀ cm³ on day 0. Control infection on day 28 after inoculation was carried out with the virulent strain of ASF virus "Stavropol 01/08" (genotype II).

Animals

The study used 4 clinically healthy Large White pigs aged 5 months and weighing 40-50 kg. The animals were randomly divided into 2 groups with 2 animals in each group and housed in separate rooms. The experimental plan with animals was approved by the Ethics Committee of the Federal Research Center for Virology and Microbiology, and permission was obtained to conduct the study (protocol 1 dated 10.02.2023). Clinical signs were assessed according to the method developed by King *et al.*, 2011 [6].

Sampling

Blood and serum samples were collected from the cranial vena cava on days 0, 3, 5, 7, 10, 14, 21, and 28 after inoculation with ASF virus "Vologograd/14c" (group "V") and "Vologograd/D(1L-5-6L) MGF110" (group "M"). After challenge with the virulent

ASF virus “Stavropol 01/08”, blood and serum samples were taken on 3, 5, 7, 10, 14, 21, 28, 35 days.

Samples of heparinized blood taken from the cranial vena cava from 20-25 kg pigs were prepared by Ficoll-Hypaque gradient (density 1.077 g/cm³, GE Healthcare, Chicago, IL, USA) with 0.1% lactalbuminhydrolyzate in Earle’s physiological solution with the addition of 10% donor pig blood serum was used as a nutrient medium (pH 7.60-7.65). The porcine blood derived macrophages was distributed into 24-48 well plastic microplates («Nunc», Roskilde, Denmark). The wells were filled with a cell suspension to achieve a concentration of 3.0 – 3.5 million cells/ml. The micropannels were incubated with the following parameters: CO₂ concentration 5%, relative humidity 90% and temperature (37.0±0.5)°C.

Detection of the ASF virus genome by real-time PCR

Total DNA extraction from the tested samples was performed using the “DNA-sorb B” kit («Amplisense», Russia) according to the manufacturer’s instructions. The presence of ASF virus genome fragments in the isolated total DNA samples was determined by real-time polymerase chain reaction (PCR) using the CFX 96 touch amplifier («BioRad», USA) according to the modified protocol recommended by OIE [7].

ELISA

Virus-specific antibodies to the ASF virus p30 protein were detected in blood serum samples using the “ASF-SEROTEST plus” enzyme immunoassay («Vetbiochem LLC», Moscow, Russia).

Determination of virus infectious activity

The ASF virus infectious activity was determined by titration in a 3-day culture of porcine blood derived macrophages. The results were evaluated based on the presence of specific hemadsorption within 7-10 days. Virus titers were calculated using the method described by B.A. Kerber modified by I.P. Ashmarin and expressed in 50% hemadsorbing units (HAU₅₀cm³) [8].

RESULTS

After immunization on day 0, pig No. 2 from the group inoculated with the “Volgograd/14c” strain (“V”) exhibited a short-term 2-day hyperthermia of 40.1 – 40.3°C on days 6-7. No other clinical signs were observed in this animal later. The other animals remained clinically healthy throughout the 28-day period until the control infection. The clinical score for all pigs after inoculation and before the control infection did not exceed 2-3 points. The temperature data of the animals during the experiment are presented in Figure 1.

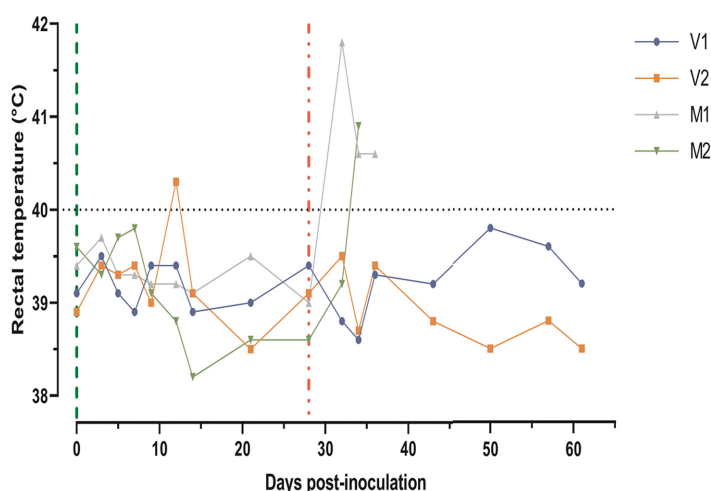


Figure 1. Temperature graph of the animals in groups «B» («Volograd/14C») and «M» («Volograd/D(1L-5-6L) MGF110»). The red dashed line indicates the timing of the control infection of immunized animals.

After the control infection with the “Stavropol 01/08” strain, animals in group “M” developed clinical signs characteristic of acute ASF on days 3-4. The disease was accompanied by hyperthermia of 40.6 – 41.8°C, refusal of feed, apathy, cyanosis of the ear tips and underbelly, difficulty breathing and hind limb paralysis for 1-2 days before death. The disease duration was 3-5 days with the animals dying on days 7 and 8, respectively. The survival data of the animals during the experiment are presented in Figure 2. The maximum clinical scores after the control infection reached 17-19 points. Post-mortem examination of the deceased animals revealed characteristic pathological changes of acute ASF, including serosanguinous and hemorrhagic lymphadenitis of the submandibular, mediastinal, portal, gastric, mesenteric, and inguinal lymph nodes. Hemorrhages on the heart, congestive hyperemia and edema of the lungs, hemorrhages in the cortical and medullary layers of the kidneys, and splenic hemorrhage were also observed.

Pig No. 1 from group “V” exhibited a rise in body temperature of 40.3 – 41.2°C on days 10-12 with subsequent minor increases on days 22, 23, and 27 after the control infection. Pig No. 2 from group “V” remained clinically healthy throughout the entire experiment (32 days). The clinical score for pigs immunized with the “Volograd/14C” strain after the control infection did not exceed 2 and 5 points for pig No. 2 and pig No. 1, respectively (Figure 1). On day 32 after the control infection, the animals were euthanized. No visible pathological changes in the internal organs were observed during the necropsy.

The results of the real-time PCR study of DNA isolated from blood samples taken during the experiment are presented in Figure 3.

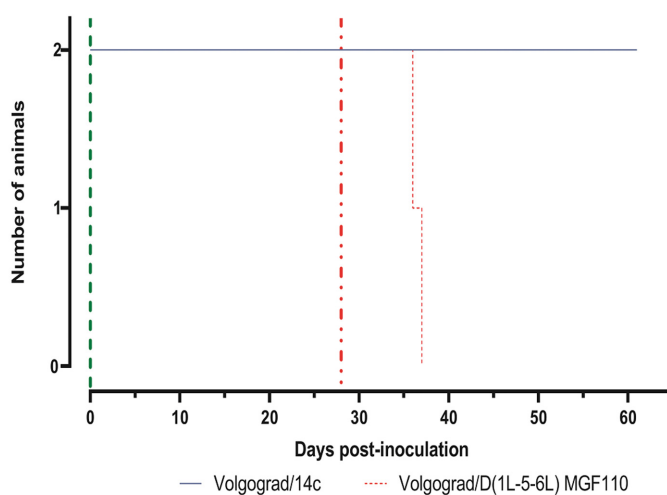


Figure 2. Survival graph of the animals during the experiment. The red dashed line indicates the timing of the control infection of immunized animals.

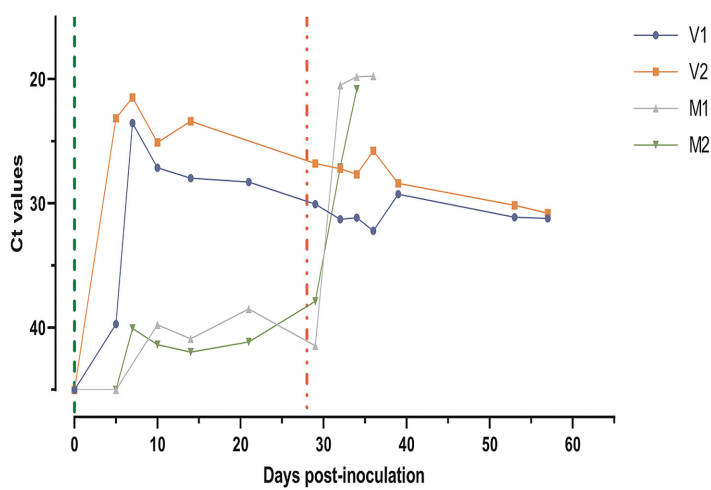


Figure 3. Ct values of DNA samples isolated from blood of groups “V” (“Volgograd/14c”) and “M” (“Volgograd/D(1L-5-6L) MGF110”). The red dashed line indicates the timing of the control infection of immunized animals.

From the graph, it can be seen that the Ct values in samples from group "V" were initially higher than those in group "M" and did not change significantly throughout the experiment. In samples collected from animals immunized with the recombinant strain "Volgograd/D(1L-5-6L) MGF110", the Ct values were significantly lower compared to group "B", but after the control infection, a significant increase was observed until the animals' death.

Investigation of serum samples collected on days 0, 3, 5, 7, 10, 14, 21, and 28 after inoculation with the "Volgograd/14c" strain allowed the detection of antibodies to the ASF virus p30 protein starting from day 10 (pig V2) and day 14 (pig V1). No antibodies were detected in serum samples from animals inoculated with the recombinant strain "Volgograd/D(1L-5-6L) MGF110".

Viremia in the blood of pigs inoculated with the ASFV "Volgograd/14c" strain was observed from 5-7 days in titers of 2.5 – 3.0 lg HAU₅₀ cm³. The maximum virus accumulation was observed on days 10-14 which reached 3.5 – 4.75 lg HAU₅₀ cm³. By days 21-28 viremia in animal No. 2 decreased, the titers were 2.5 – 3.25 lg HAU₅₀ cm³, while in pig No. 1 the virus was not detected on days 21 and 28 after infection. In animals of group No. 2, after immunization by the ASFV "Volgograd/D(1L-5-6L) MGF110" the infectious virus was not detected in the blood for 28 days (observation period) (Table 1).

Table 1 – Virus titer in the blood of pigs immunized with the ASF virus "Volgograd/14c" and its deletion variant "Volgograd/D(1L-5-6L) MGF110". The virus titers in the samples are indicated in lg HAU₅₀ cm³.

Day	Group 1 (“Volgograd/14c”)		Group 2 (“Volgograd/D(1L-5-6L) MGF110”)	
	Animal №1	Animal №2	Animal №1	Animal №2
0	0	0	0	0
3	0	0	0	0
5	0	3.0	0	0
7	2.5	3.5	0	0
10	3.5	4.25	0	0
14	3.0	4.75	0	0
21	0	3.25	0	0
28	0	2.5	0	0

DISCUSSION

The main method for elucidating the specific functions of ASFV genes is the development of deletion viruses. The scientific literature contains a wealth of data indicating the role of certain genes in the ASFV biology and describing their impact. [9;10]. However, there is relatively limited information available on the functions of genes belonging to multigene families in the ASFV genome. According to the available data, the deletion of MGF360-9L and MGF505-7R genes contributes to virus attenuation, and immunization with the deletion virus protecting against infection with the parental strain [11]. The deletion of the MGF100-1R gene does not lead to changes in the properties of the ASFV strain isolated in China [12]. As for MGF 110, studies have shown that the 9L gene deletion also contributes to the virus attenuation [13].

In the work of Netherton et al., 2004 it was noted that the products of the MGF 110-4L and – 6L genes are localized in pre-Golgi compartments and are involved in endoplasmic reticulum remodeling, disrupting its ability to synthesize proteins that stimulate cytokine production or participate in antigen presentation. Therefore, they do not affect the virus's ability to replicate in pig organisms but rather disrupt the immune response [14]. This is further supported by the study by Ren et al., 2023 which investigated the impact of the 9L gene deletion which is part of MGF 110, by comparing the properties of the parental and deletion strains. The study revealed the 9L gene's influence on the degradation of TANK Binding Kinase 1 (TBK1), which affects the type I interferon production. The parental strain inhibits type I interferon production by degrading TBK1, while the deletion strain enhances type I interferon production by weakening TBK1 recovery, clarifying the mechanism by which the 9L gene deletion of MGF 110 ensures attenuated virulence *in vitro* [15,16]. The deletion of the 11L gene of MGF110 has a weak effect on virus virulence, increases the induction of an immune response, and causes mild clinical signs [17]. The 7L gene influence was studied in the work of Zhong et al., 2022. It was shown that MGF110-7L suppresses translation processes in infected cells and contributes to the stress granules formation. This occurs due to the phosphorylation of the eukaryotic initiation factor 2 α (eIF2 α) signaling pathway by Protein Kinase R (PKR) and PKR-like endoplasmic reticulum kinase (PERK) [18].

In a previous study, the influence of the deletion of the 1L-5-6L MGF110 gene fragment on virus replication in COS-1 cells and its growth characteristics were investigated [19]. It was found that these properties did not change during the cultivation of the recombinant strain “Volgograd/D(1L-5-6L) MGF110” in COS-1 cells. The data obtained in *in vitro* models do not always correspond to how the virus will manifest itself in the susceptible animal body. Based on the data obtained in this study, it can be concluded that there is a change in the accumulation level of the recombinant strain compared to the parental strain *in vivo*. This is indicated by significantly lower Ct values obtained when investigating DNA samples isolated from blood collected from

animals during immunization before the control infection with the virulent ASFV strain "Stavropol 01/08". However, after the control infection, a significant increase is observed in the Ct values of samples from animals inoculated with the "Volgograd/D(1L-5-6L) MGF110" strain (group "M") leading to death while Ct values in samples from animals inoculated with the parental strain "Volgograd/14c" (group "V") did not change significantly until the experiment end. This may indicate stable persistence of the parental strain "Volgograd/14c" in the animals' bodies.

These results are confirmed by the investigation of serum samples using the ELISA method, as well as the determination of the virus infectious activity in blood samples from immunized animals. The presence of antibodies to the p30 protein in serum samples and the active virus presence in blood samples from animals in group "V" confirm the reproduction of the virus strain "Volgograd/14c" in the animals' bodies. However, no antibodies or active virus of the deletion strain "Volgograd/D(1L-5-6L) MGF110" were detected in serum and blood samples from animals in group "M" indicating the absence of virus replication.

The results obtained in this experiment differ from those published by Ramirez-Medina *et al.*, 2022 [20]. In their study, the authors investigated the properties of the deletion strain "ASFV-G-ΔI177L/ΔMGF110-5L-6L" in which the I177L and MGF110-5L-6L genes are deleted. The authors claim that the MGF110-5L-6L deletion has no effect on the virus properties and growth characteristics both *in vitro* and *in vivo* and can serve as a DIVA marker for a candidate vaccine strain. Such discrepancies in the results can be explained by the fact that Ramirez-Medina *et al.*, 2022 used the ASF virus virulent strain "Georgia" (ASFV-G), which is closest to the strains currently circulating in Europe and the Russian Federation. Furthermore, the strain they used was not adapted to immortalized cell lines and only replicated in primary pig leukocyte cell culture. In our study, the "Volgograd/14c" strain was used, which had undergone several passages on cell cultures, including immortalized cell lines, resulting in its partial attenuation. It is likely that the observed changes in properties exhibited by the virus in *in vitro* or *in vivo* conditions may be due not only to the specific gene deletion but also to a combination of changes that occurred in the virus genome as a result of adaptation to immortalized cell lines. Additionally, the "Volgograd/D(1L-5-6L) MGF110" strain had a larger deletion encompassing genes from 1L to 5-6L.

CONCLUSIONS

The experiment on susceptible animals revealed that the recombinant strain with a deletion of genes part from the multigene family 110 does not replicate in pig organisms. However, as shown in a previous study, there are no significant differences in growth characteristics and accumulation between the parental and deletion strains. Based on this, it can be concluded that the 1L-5-6L genes of the multigene family 110 play a crucial role in the virus's adaptation to replicate in susceptible animal organisms.

Acknowledgements

This work was supported by the Russian Science Foundation, project № 22-24-00552.

Ethics approval

Authors confirm compliance with institutional and national standards for the use of laboratory animals in accordance with «Consensus Author Guidelines for Animal Use» (IAVES, 23 July 2010). The research protocol was approved by the Ethics Committee of the institution (Protocol No 1 dated 10.02.2023).

Authors' contributions

MEV made animal immunization, blood and serum samples collection, data collection analysis, interpretation, and manuscript preparation. MVN prepared porcine blood-derived macrophages, data collection, ELISA, and writing – original draft preparation. DAK made data analysis and manuscript preparation. IAT creates study design and conceptualization, PCR experiments, data analysis, writing – review, and editing. All authors have read and agreed to the published version of the 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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ODREĐIVANJE UTICAJA 1L-5-6L MGF110 GENA NA BIOLOŠKA SVOJSTVA VIRUSA AFRIČKE SVINSKE KUGE (*ASFARVIRIDAE*; *ASFIVIRUS*) "VOLGOGRAD/14C" *IN VIVO*

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Afrička kuga svinja i dalje predstavlja globalni problem zbog odsustva prevencije vakcinom i visoke cene antiepizootskih mera. Ova studija ispituje funkcionalnu ulogu virusa afričke svinjske kuge (ASFV) 1L-5-6L multigenske porodice 110 gena *in vivo*. U ovoj studiji su korišćene četiri klinički zdrave Velike Bele svinje. Dve grupe životinja su inokulisane ili sa roditeljskim sojem ili sa delecijom varijantom. Za naknadnu izazovnu infekciju korišćen je homologni virulentni soj "Stavropol 01/08".

Uzorci krvi su sakupljeni u određenim vremenskim intervalima. Infektivna aktivnost ASFV je određena titracijom u makrofagima svinjske krvi. Antitela specifična za virus na ASFV p30 protein su otkrivena korišćenjem enzimskog imunosorbentnog testa (ELISA).

Rezultati lančane reakcije polimeraze (PCR) u realnom vremenu pokazali su značajnu razliku u vrednostima Ct između uzoraka iz dve grupe životinja. Određivanje infektivne aktivnosti ASFV u uzorcima krvi pokazalo je prisustvo virusa kod životinja imunizovanih roditeljskim sojem. Virus nije detektovan u uzorcima životinja imunizovanih delecijom sojem. ELISA metoda je pokazala prisustvo antitela na protein p30 u uzorcima seruma od 10 do 14 dana nakon imunizacije roditeljskim sojem, dok u uzorcima seruma životinja imunizovanih delecijom sojem nisu otkrivena antitela.

Osobine rekombinantnog soja ASFV „Volvograd/D(1L-5-6L) MGF110” proučavane su u *in vivo* ogledu. Utvrđeno je da se delecijski soj ne reprodukuje kod životinja, za razliku od roditeljskog soja.