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THE STUDY OF ANTIGENICITY, IMMUNOGENICITY AND PROTECTIVE POTENTIAL OF DNA CONSTRUCTS CONTAINING FRAGMENTS OF GENES CP204L, E183L OR EP402R OF AFRICAN SWINE FEVER VIRUS STRAIN MK-200

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Abstract

African swine fever (ASF) is a viral, contagious and septic disease affecting wild and domestic pigs of all breeds and age groups. In both domestic pigs and wild European boars affected, some hyperacute or acute forms of the infection are observed which are characterized by fever, signs of toxicosis, hemorrhagic diathesis with mortality rates of up to 100 %. In endemic regions (e.g., some countries of East Africa), a subacute form of the disease with a mortality of 30 to 70 %, as well as a chronic one with very low mortality levels are reported (S. Blome et al., 2013; C. Gabriel et al., 2011; JM Sánchez -Vizcaíno et al., 2015). No vaccine against African swine fever is currently available, and the research works aimed at the development of live, inactivated and subunit vaccines have not achieved the intended result yet (P.J. Sánchez-Cordón et al., 2017; V. O'Donnell et al., 2016; S. Blome et al., 2014). Nevertheless, the opportunity of obtaining a DNA vaccine using the genes of potentially protective ASFV proteins p30, p54 and/or CD2v is considered to be a promising option in a number of laboratories worldwide. The proteins p30 and p54 are functionally important in attaching the ASF virus to the target cell. The antibody to p54 blocks the virion binding to the macrophage, whereas the antibody to p30 inhibits the virion penetration into the cell. The protein CD2v determines the hemadsorbing properties of the virus (S.D. Kollnberger et al., 2002; M.G. Barderas et al., 2001; J.G. Neilan et al., 2004). This work has been performed to study effects of the translation products of recombinant plasmids pCI-neo/ASFV/p30, pCI-neo/ASFV/p54 and pCI-neo/ASFV/CD2v induced in adhesive cells (A-cells) after transfection. The antigenic activity of the recombinant proteins produced with these DNA constructs was compared in permanent cell line HEK-293T and swine leukocyte (SL) autologous primary cultures using a direct fluorescence technique. The highest expression of the antigen-active translation products in HEK-293T and SL cells transfected with pCI-neo/ASFV/p30, pCI-neo/ASFV/p54 or pCI-neo/ASFV/CD2v was observed on day 2. The peculiarity of the pig immunization schedule applied was that the animals were thrice immunized at a 14-day interval with autologous LS A cells transfected in vitro with the above recombinant plasmids. For this, as much as 90 cm^3 of LC cell culture was produced using blood samples from each of the animals No.No. 1-4. On day 2 of the culturing, 90 μg of pCI-neo/ASFV/p30 (No. 1), pCI-neo/ASFV/p54 (No. 2) or pCI-neo/ASFV/CD2v (No. 3) was added thereto. The LS culture obtained from the pig No. 4 in a volume of 90 cm^3 was divided into three portions of 30 cm^3, and each one was transfected with one of the three constructs (i.e., pCI-neo/ASFV/p30, pCI-neo/ASFV/p54 or pCI-neo/ASFV/CD2v) by adding 30 μg of the plasmid DNA. Two days later, the pigs No. 1 to No. 4 were inoculated into the central auricular vein with about 10^7 autologous transfected A-cells of LS cultures. On days 14, 28 and 42, no antibody against ASFV proteins was detected in the blood of the immunized pigs using indirect solid-phase ELISA and immunoblotting. After the pigs were infected into the neck with 10^2 HAU_{50} of an ASFV strain Mozambique-78 on day 42, the four pre-immunized pigs (No.No. 1-4) died on day 6 to 8 and the control one died (No. 5) on day 13. Thus, the immunization of pigs with the autologous and antigenically active

LS cells transfected with the recombinant plasmids pCI-neo/ASFV/p30, pCI-neo/ASFV/p54 or pCI-neo/ASFV/CD2v failed to provide antibody response or protection against the challenge.

Keywords: African swine fever, ASFV, recombinant plasmids, ASFV proteins p30, p54 and CD2v, antigenicity, immunogenicity

African swine fever (ASF) is a viral contagious septic disease affecting wild and domestic pigs of all breeds and age groups. In domestic pigs and wild European boars affected, hyperacute or acute course of disease with fever, toxicosis, hemorrhagic diathesis and mortality rates of up to 100% are observed. In endemic regions (some countries of East Africa), a subacute form of the disease with the mortality from 30 to 70%, as well as a chronic form with very low mortality [1-3] are registered. The researches on the development of live inactivated and subunit vaccines have not yet yielded the desired results (4-6). Certain hopes are placed on the construction of recombinant DNA vaccines (7, 8).

It is likely that in case of ASF infection the formation of specific protection is ensured by the set of proteins inducing the immunological protection which is ensured by both cytotoxic T-lymphocytes and antibody-dependent cellular cytotoxicity [9-11]. Based on the data on the localization, structure and functional properties of viral proteins, polypeptide specificity of antibodies in the pigs' blood serum of after the administration of attenuated or virulent strains of ASF virus (ASFV), and on the effects of pigs immunization with the proteins extracted from infected cells or with recombinant proteins, p30, p54 and CD2v proteins are considered as potentially protective ones. The p30 and p54 proteins are functionally important for attaching ASFV to the target cell. The antibodies to p54 block the binding of the virion to the macrophage, while the antibodies to p30 inhibit the virion's penetration into the cell. CD2v protein ensures hemadsorbing activity of the virus [12-14].

Study of the immunogenic and protective properties of DNA constructs containing the genes of the viral proteins p30, p54 and CD2v confirmed the important role of cellular immunity in the formation of specific protection against ASF that opens up the prospects for developing new-generation drugs [15-17].

We previously reported on the obtaining of pCI-neo/ASFV/p30, pCI-neo/ASFV/p54 and pCI-neo/ASFV/CD2v recombinant plasmids. The calculated molecular weights of unmodified recombinant proteins amounted to 21.6 kDa (rp30), 18.7 kDa (rp54) and 28.6 kDa (rCD2v). According to the results of immunoblotting, in the continuous cell culture HEK-293T the molecular weight of the antigenically active products of translation of pCI-neo/ASFV/p30 amounted to 21.6 kDa, pCI-neo/ASFV/p54 – 20.9 kDa and 36.3 kDa [18]. According to the data of P. Gymez-Puertas et al. [19] and F. Rodriguez et al. [20], the weight of monomer of full-size p54 is 24-28 kDa. In the HEK-293T cells transfected by pCI-neo/ASFV/CD2v the translated virus-specific polypeptides had a molecular weight of 28.8; 39.8; 63.1 and 104.7 kDa. The first of them by its size corresponded to the calculated unmodified molecule of rCD2v. The others, apparently, were the forms modified at different degrees in the process of glycosylation and trimming. These results mainly correspond to the data of L.C. Goatley and L.K. Dixon [21], who identified in Vero cells transfected by the SV5CD2vHA plasmid the polypeptides of recombinant CD2v with the molecular weights of 26; 63; 89 and 104 kDa.

In the presented work, we immunized the animals with the A-cells of autologous LC culture transfected in vitro by recombinant plasmids for increasing the efficiency of induction of anti-cellular mechanisms of protection against ASF, however, as it turned out, we couldn't achieve the protective effect.

The objective of our researches is to determine the antigenicity, immu-

nogenicity and protectivity of the products of translation of pCI-neo/ASFV/p30, pCI-neo/ASFV/p54 and pCI-neo/ASFV/CD2v recombinant plasmids.

Techniques. The domestic pigs of the Large White breed with the weight of 20-25 kg were from the experimental sector of the Federal Research Center of Virology and Microbiology (FRCVM). We used the continuous cell line NEK-293T (Human Embryonic Kidney 293) from the Museum of Cells Culture of the FRCVM and the obtained primary culture of pig leukocytes. The virulent strain ASFV Mozambique-78 (M-78) and its attenuated derivative MK-200 strain were taken from the State Collection of Microorganisms of the FRCVM [22].

In order to prepare the LC culture, the blood sample (30-40 ml) was taken from the vena cava cranialis of each animal; heparin (20 units/cm^3) was used to stabilize the blood. The blood was kept for 50-60 min at 37 °C and then the upper fraction consisting of plasma and leukocytes was collected and centrifuged at 2000 g for 15 min, the supernatant was removed, and the sediment was resuspended to obtain the inoculum of leukocytes (4 million per cm^3) in Eagle's MEM medium with 10% homologous blood serum inactivated for 30 min at 56 °C, penicillin (100-200 U/cm^3) and streptomycin (100-200 mg/cm^3). The cells were cultured in 6-well plates in CO_2-incubator at 37 °C. One hour prior to the transfection, 2-day LC cell culture was transferred to serum-free medium.

The cells were transfected with DNA constructs for 6-8 hours with the use of Lipofectamine® (Thermo Fisher Scientific, United States) according to the standard protocol. Then, the culture medium was replaced with the Eagle's MEM medium with 10% homologous swine serum and incubated for 2-3 days.

The anti-ASFV positive sera were obtained according to the following scheme. The ASFV strain MK-200 at a dose of 6.5 lg HAU_{50} (day 0) was injected intramuscularly to domestic pigs. On day 17, these pigs were infected intramuscularly with ASFV strain M-78 at a dose of 10^3 HAU_{50}. During the periods of clinical signs of disease (hyperthermia, refusal of food, hemorrhages on the ears and belly) the sick animals were treated with daily intramuscular injection of phosphonoacetic acid (100 mg/kg) until the restoration of normal body temperature (3-5 days). The total exsanguination of pigs was performed on day 38 after the start of the experiment.

The obtaining of the immunoglobulins marked with fluorescein isothiocyanate (FITC) from the sera of ASF-resistant pigs (anti-ASFV FITC immunoglobulins) and conducting the reaction of direct immunofluorescence were performed according to State Standard of the Russian Federation GOST 28573-90 "Pigs. Methods of Laboratory Diagnosis of African Swine Fever". The results were documented by fluorescence microscopy (Eclipse E200 fluorescence microscope, Nikon Corp., Japan). The pig blood sera were examined for the presence of antibodies to ASFV proteins using indirect enzyme-linked immunosorbent assay (ELISA) and immunoblotting [23, 24] methods.

Results. In our work, we used previously obtained expressing DNA constructs (pCI-neo/ASFV/p30, pCI-neo/ASFV/p54, pCI-neo/ASFV/CD2v) with fragments of the CP204L, E183L and EP402R ASFV genes of the MK-200 strain of seroimmunotype III [18].

The antigenic activity of recombinant proteins produced by these DNA constructs was studied in HEK-293T and LC cell cultures. The coverslips with transfected cells were taken daily and the production of antigenically active recombinant proteins was determined by direct immunofluorescence method. The maximum expression in HEK-293T and LC cells transfected by pCI-neo/ASFV/p30, pCI-neo/ASFV/p54 or pCI-neo/ASFV/CD2v was observed on days 2-3 (Fig. 1).

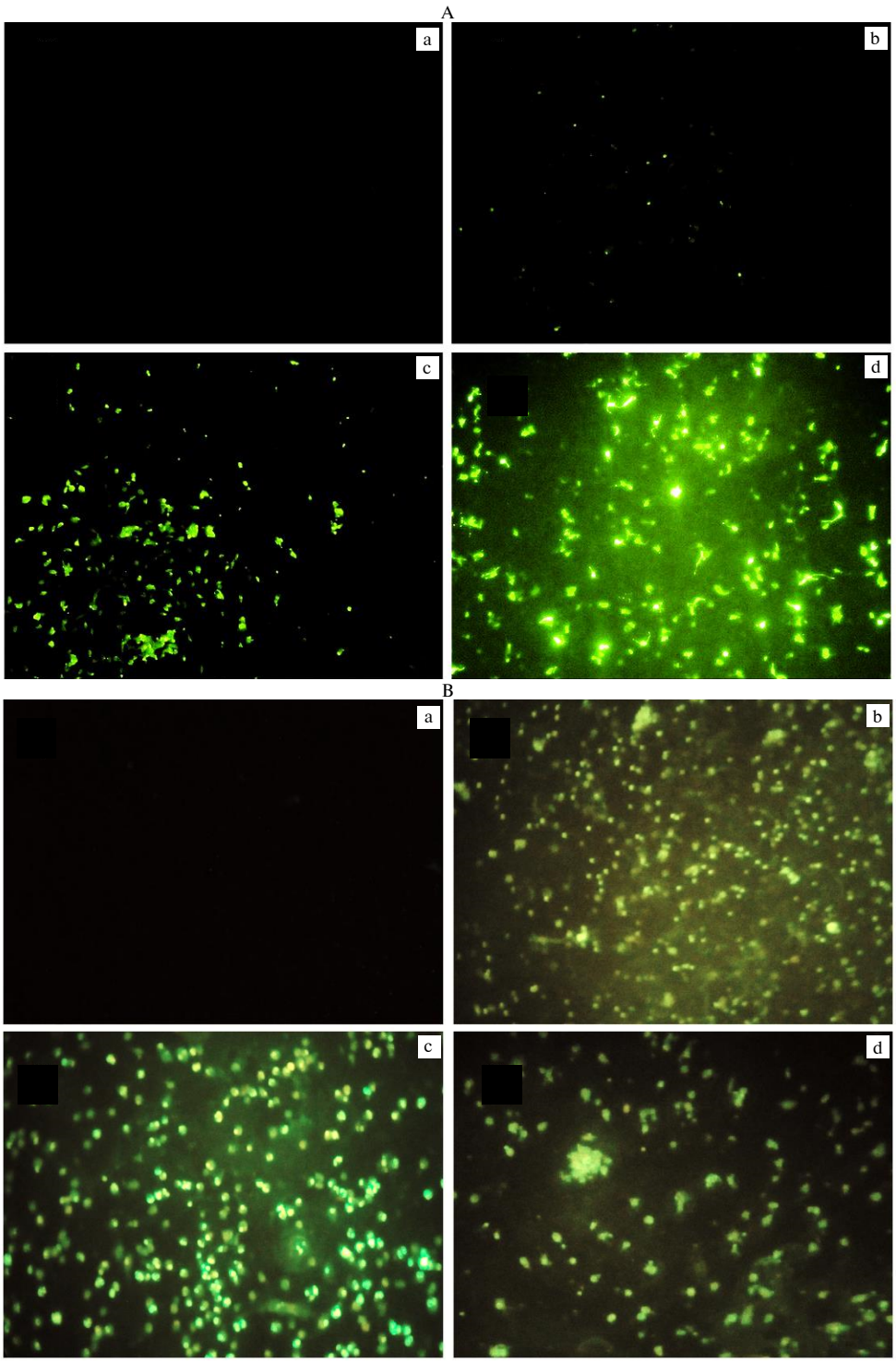

Fig. 1. Expression of antigenically active products in HEK-293T continuous cell line (A) **and in primary culture of pig leukocytes** (B) **transfected by pCI-neo/ASFV/p30 recombinant plasmid**: a, b, c, d — respectively 0, 1, 2, 3 days after the transfection (fluorescent microscopy, Eclipse E200, Nikon Corp., Japan, magnification ×100).

The peculiarity of the pig immunization scheme was that it was performed on days 0, 14 and 28 with using A cells of the autologous LC culture transfected in vitro with the recombinant plasmids. It was supposed that the im-

munization with antigen-presenting cells (macrophages) will ensure the effective induction of anti-cellular mechanisms of protection against ASF. For this, 4 days prior the immunization, LC cell cultures (90 cm^3) of pigs No. 1-4 was obtained. On day 2 of culture, 90 μg of pCI-neo/ASFV/p30 (No. 1), pCI-neo/ASFV/p54 (No. 2) or pCI-neo/ASFV/CD2v (No. 3) were introduced; 90 cm^3 LC culture of pig No. 4 was divided into three parts by 30 cm^3 and each portion was transfected with one of three constructs (30 μg pCI-neo/ASFV/p30, pCI-neo/ASFV/p54 or pCI-neo/ASFV/CD2v). After 2 days, the culture media with non-attached LC cells were removed, and the monolayer of transfected cells (about 10^7 A-cells) was mechanically taken off the substrate, washed at 2000 g for 10 min, resuspended in 3 ml phosphate buffer (pH 7.2) and injected into central ear vein to the relevant autologous pigs. The animals were clinically observed with daily measurement of body temperature. Using indirect ELISA and immunoblotting, we have not detected in the blood of immunized pigs the antibodies to ASFV proteins on days 0, 14, 28 and 42. After the control infection with M-78 ASFV strain (10^2 HAU_{50}) in the neck on day 42, four immunized pigs died in 6-8 days, and non-immunized (control) pig died in 13 days (Fig. 2).

The pig which was immunized with A-cells of LC culture transfected with the pCI-neo/ASFV/p30 construct (No. 1) died after 6 days without any clinical and pathoanatomical signs. Other immunized animals showed the increase in body temperature and died on days 7-8 with the characteristic pathoanatomical signs of ASF [25]. The control animal (No. 5) showed loss of appetite, depressed state, fever (body temperature up to 41.2-41.3 °C), paresis and paralysis of the hind limbs. Cyanosis occurred on skin of ears, belly, limbs and perineum. The pathoanatomical presentation was typical for ASF.

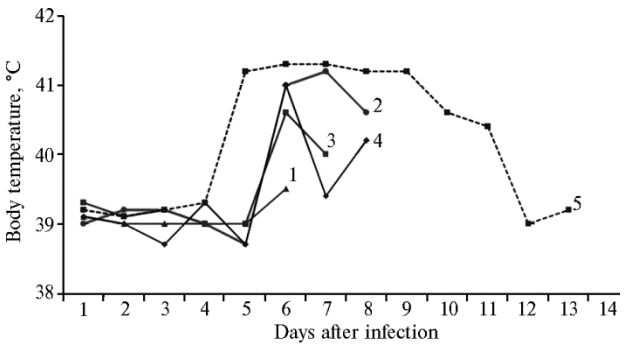

Fig. 2. Body temperature of pigs immunized with various recombinant ASFV proteins (1-4) **for the period from infection to death, and of the control animal** (5) **after the experimental infection with the virulent ASFV Mozambique-78 strain**: 1 — p30, 2 — p54, 3 — CD2v, 4 — p30, p54 and CD2v.

The significant shortcoming of candidate DNA vaccines is the relatively low induction of immune responses, especially in large mammals. In order to overcome this problem for ASF, several approaches have been tested: targeting, ubiquitination, immunization with expression libraries, s well as BacMam viruses [26]. The first attempts to induce the protective immune response against ASFV using the DNA construct encoding two viral proteins p54 and p30 as a chimeric protein (PQ) were unsuccessful. The DNA constructs encoding only PQ ensured high production of antibodies in immunized mice, but not in pigs [8].

The immunization with the pCMV-sHAPQ DNA construct supplemented with the gene for CD2v protein (HA) induced in pigs the humoral immune response, but the pigs were not protected from the control introduction of infection and showed the clinical signs of ASF and viremia kinetics which were indistinguishable from those in control animals. In order to avoid the unwanted induction of antibodies and to enhance the specific $CD8^+$-T-cell responses, the pCMV-UbsHAPQ construct encoding p30, p54 and CD2v antigenic determinants joined with cell ubiquitin (Ubs) has been developed. The twice done immunization with pCMV-UbsHAPQ has not induced the humoral response in

pigs, but ensured the partial protection against control infection with ASFV, thereby confirming the importance of the T-cell response in protection against this virus. The four-time immunization with pCMV-UbsHAPQ stimulated the formation of antibodies to p30 and p54 that apparently resulted in the decrease of the protective effect of $CD8^+$-T-cells [16]. It is possible that the earlier, as compared to the control animal, death of the immunized pigs in our experiment was due to the induction of virus-specific antibodies in titers insufficient for detection by indirect ELISA and immunoblotting methods.

Thus, expression of ASFV-specific products in HEK-293T cells and leukocytes of pigs transfected with the pCI-neo/ASFV/p30, pCI-neo/ASFV/p5 and pCI-neo/ASFV/CD2v recombinant plasmids has been proved by immunofluorescence method. The immunization of pigs with the autologous porcine leukocytes transfected with the pCI-neo/ASFV/p30, pCI-neo/ASFV/p54 or pCI-neo/ASFV/CD2v constructs has not induced the virus-specific antibodies and protection against the control infection.

REFERENCES

1. Blome S., Gabriel C., Beer M. Pathogenesis of African swine fever in domestic pigs and European wild boar. *Virus Res.*, 2013, 173(1): 122-130 (doi: 10.1016/j.virusres.2012.10.026).
2. Gabriel C., Blome S., Malogolovkin A., Parilov S., Kolbasov D., Teifke J.P., Beer M. Characterization of African swine fever virus Caucasus isolate in European wild boars. *Emerg. Infect. Dis.*, 2011, 17(12): 2342-2345 (doi: 10.3201/eid1712.110430).
3. Sánchez-Vizcaíno J.M., Mur L., Gomez-Villamandos J.C., Carrasco L. An update on the epidemiology and pathology of African swine fever. *J. Comp. Pathol.*, 2015, 152(1): 9-21 (doi: 10.1016/j.jcpa.2014.09.003).
4. Sánchez-Cordón P.J., Chapman D., Jabbar T., Reis A.L., Goatley L., Netherton C.L., Taylor G., Montoya M., Dixon L. Different routes and doses influence protection in pigs immunised with the naturally attenuated African swine fever virus isolate OURT88/3. *Antiviral Res.*, 2017, 138: 1-8 (doi: 10.1016/j.antiviral.2016.11.021).
5. O'Donnell V., Holinka L.G., Sanford B., Krug P.W., Carlson J., Pacheco J.M., Reese B., Risatti G.R., Gladue D.P., Borca M.V. African swine fever virus Georgia isolate harboring deletions of 9GL and MGF360/505 genes is highly attenuated in swine but does not confer protection against parental virus challenge. *Virus Res.*, 2016, 221: 8-14 (doi: 10.1016/j.virusres.2016.05.014).
6. Blome S., Gabriel C., Beer M. Modern adjuvants do not enhance the efficacy of an inactivated African swine fever virus vaccine preparation. *Vaccine*, 2014, 32(31): 3879-3882 (doi: 10.1016/j.vaccine.2014.05.051).
7. Lacasta A., Ballester M., Monteagudo P.L., Rodríguez J.M., Salas M.L., Accensi F., Pina-Pedrero S., Bensaid A., Argilaguet J., Lypez-Soria S., Hutet E., Le Potier M.F., Rodríguez F. Expression library immunization can confer protection against lethal challenge with African swine fever virus. *J. Virol.*, 2014, 88(22): 13322-13332 (doi: 10.1128/JVI.01893-14).
8. Argilaguet J.M., Pérez-Martín E., Nofrarías M., Gallardo C., Accensi F., Lacasta A., Mora M., Ballester M., Galindo-Cardiel I., Lypez-Soria S., Escribano J.M., Reche P.A., Rodríguez F. DNA vaccination partially protects against African swine fever virus lethal challenge in the absence of antibodies. *PLoS ONE*, 2012, 7(9): e40942 (doi: 10.1371/journal.pone.0040942).
9. Sereda A.D., Kazakova A.S., Imatdinov A.R., Kolbasov D.V. Humoral and cell immune mechanisms under African swine fever (review). *Sel'skokhozyaistvennaya Biologiya* [*Agricultural Biology*], 2015, 50(6): 709-718 (doi: 10.15389/agrobiology.2015.6.709eng).
10. Lacasta A., Monteagudo P.L., Jiménez-Marín Б., Accensi F., Ballester M., Argilaguet J., Galindo-Cardiel I., Segalés J., Salas M.L., Domínguez J., Moreno Б., Garrido J.J., Rodríguez F. Live attenuated African swine fever viruses as ideal tools to dissect the mechanisms involved in viral pathogenesis and immune protection. *Vet. Res.*, 2015, 46: 135 (doi: 10.1186/s13567-015-0275-z).
11. Takamatsu H.H., Denyer M.S., Lacasta A., Stirling C.M., Argilaguet J.M., Netherton C.L., Oura C.A., Martins C., Rodríguez F. Cellular immunity in ASFV responses. *Virus Res.*, 2013, 173(1): 110-121 (doi: 10.1016/j.virusres.2012.11.009).
12. Kollnberger S.D., Gutierrez-Castaceda B., Foster-Cuevas M., Corteyn A., Parkhouses R.M.E. Identification of the principal serological immunodeterminants of African swine fever virus by screening a virus cDNA library with antibody. *J. Gen. Virol.*, 2002, 83: 1331-1342 (doi: 10.1099/0022-1317-83-6-1331).
13. Barderas M.G., Rodriguez F., Gomez-Puertas P., Aviles M., Beitia F., Alonso C., Escribano J.M.

Antigenic and immunogenic properties of a chimera of two immunodominant African swine fever virus proteins. *Arch. Virol.*, 2001, 146: 1681-1691 (doi: 10.1007/s007050170056).
14. Neilan J.G., Zsak L., Lu Z., Burrage T.G., Kutish G.F., Rock D.L. Neutralizing antibodies to African swine fever virus proteins p30, p54, and p72 are not sufficient for antibody-mediated protection. *Virology*, 2004, 319: 337-342 (doi: 10.1016/j.virol.2003.11.011).
15. Ruiz-Gonzalvo F., Rodríguez F., Escribano J.M. Functional and immunological properties of the baculovirus-expressed hemagglutinin of African swine fever virus. *Virology*, 1996, 218: 285-289 (doi: 10.1006/viro.1996.0193).
16. Takamatsu H.H., Denyer M.S., Lacasta A., Stirling C.M., Argilaguet J.M., Netherton C.L., Oura C.A., Martins C., Rodríguez F. Cellular immunity in ASFV responses. *Virus Res.*, 2013, 173(1): 110-121 (doi: 10.1016/j.virusres.2012.11.009).
17. Argilaguet J.M., Pérez-Martín E., López S., Goethe M., Escribano J.M., Giesow K., Keil G.M., Rodríguez F. BacMam immunization partially protects pigs against sublethal challenge with African swine fever virus. *Antiviral Res.*, 2013, 98(1): 61-65 (doi: 10.1016/j.antiviral.2013.02.005).
18. Imatdinov A.R., Sereda A.D., Imatdinov I.R., Kazakova A.S., Dubrovskaya O.A., Kolbasov D.V. Expression of recombinant genes encoding fragments of the protective important proteins of African swine fever virus in eucaryotic cells. *Sel'skokhozyaistvennaya Biologiya* [*Agricultural Biology*], 2016, 51(6): 837-844 (doi: 10.15389/agrobiology.2016.6.837eng).
19. Gómez-Puertas P., Rodriguez F., Oviedo J.M., Brun A., Alonso C., Escribano J.M. The African swine fever virus proteins p54 and p30 are involved in two distinct steps of virus attachment and both contribute to the antibody-mediated protective immune response. *Virology*, 1998, 243: 461-471 (doi: 10.1006/viro.1998.9068).
20. Rodriguez F., Alcaraz S., Yanez R.J., Rodriguez J.M., Alonso C., Rodriguez J.F., Escribano J.M. Characterization and molecular basis of heterogeneity of the African swine fever virus envelope protein p54. *J. Virol.*, 1994, 68(11): 7244-7252.
21. Goatley L.C., Dixon L.K. Processing and Localization of the African swine fever virus CD2v transmembrane protein. *J. Virol.*, 2011, 85(7): 3294-3305 (doi: 10.1128/jvi.01994-10).
22. Malogolovkin A., Burmakina G., Titov I., Sereda A., Gogin A., Baryshnikova E., Kolbasov D. Comparative analysis of African swine fever virus genotypes and serogroups. *Emerg. Infect. Dis.*, 2015, 21(2): 312-315 (doi: 10.3201/eid2102.140649).
23. Strizhakova O.M., Lyska V.M., Malogolovkin A.S., Novikova M.B., Sidlik M.V., Nogina I.V., Shkaev A.E., Balashova E.A., Kurinnov V.V., Vasil'ev A.P. Validation of an ELISA kit for detection of antibodies against ASF virus in blood or spleen of domestic pigs and wild boars. *Sel'skokhozyaistvennaya Biologiya* [*Agricultural Biology*], 2016, 51(6): 845-852 (doi: 10.15389/agrobiology.2016.6.845eng).
24. *Immunoblotting OIE for serological diagnosis of African swine fever (SOP/CISA/ASF/IB/1/2008).* Available http://asf-referencelab.info/asf/images/files/SOPs/SOP-AFSIB12008.pdf. No date.
25. Ryzhova E.V., Belyanin S.A., Kolbasov D.V., Balyshev V.M., Kurinnov V.V., Pronin V.V., Korneva G.V. *Rossiiskii veterinarnyi zhurnal*, 2012, 1: 10-14 (in Russ.).
26. Jancovich J.K., Chapman D., Hansen D.T., Robida M.D., Loskutov A., Craciunescu F., Borovkov A., Kibler K., Goatley L., King K., Netherton C,L., Taylor G., Jacobs B., Sykes K., Dixon L.K. Immunization of pigs by DNA prime and recombinant vaccinia virus boost to identify and rank African swine fever virus immunogenic and protective proteins. *J. Virol.*, 2018, 92(8): e02219-17 (doi: 10.1128/JVI.02219-17).