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EFFECT OF p30 RECOMBINANT PROTEIN ON AFRICAN SWINE FEVER VIRUS *IN VITRO* REPRODUCTION

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SUMMARY

African swine fever specific prevention means have not been developed yet. However, it is necessary to study the function of definite viral proteins, their role in immune response morphogenesis and induction to determine the components to be included into ASF protection drugs. It was established that p54 and p30 proteins participate in virus penetration and internalization and are able to induce protective antibodies in immunized pigs. The inoculation of these proteins into ASFV-infected cell culture has an impact on virus reproduction to different extents. The results of the study of purified recombinant protein p30 effect, derived from *E. coli* clone, containing pET32b(+)/p30 plasmid, on ASFV *in vitro* reproduction are presented. The greatest decrease, including complete inhibition of virus reproduction, was observed when 300 ng of p30 were inoculated into porcine spleen and marrow primary cell cultures, infected with the ASFV Krasnodar 07/17 isolate at the dose of 100 HAU per plate (~ 0.01 HAU per cell). It was noted that if the mixture of p30 and p54 was inoculated into a sample, the virus reproduction was greater compared to the use of only p30.

Key words: African swine fever, cloning, p30 recombinant protein, *in vitro* virus reproduction.

INTRODUCTION

In recent years a large number of research groups have been working hard to create means of specific prophylaxis for African swine fever (ASF) based on a live attenuated, inactivated virus, recombinant, subunit or DNA vaccine. Taking into account the results of studying the protective properties of the developed experimental vaccines, none of the preparations for commercial production received a positive assessment of its potential.

However, the development of a subunit vaccine using recombinant proteins and DNA vaccines remains one of the most topical approaches for creating environmentally safe means of specific prophylaxis for ASF. The results of the experiments showed that to determine the composite components that can be used to create reliable protection against ASF, it is necessary to study the function of a specific protein of the virus, its role in morphogenesis and inducing an immune response.

In 1998 P. Gómez-Puertas et al. established that p54 and p30 proteins of the ASF virus are able to induce protective antibodies in immunized pigs. In the course of research it was determined that p54 is responsible for the specific binding of viral particles to the macrophage and their penetration into the cell, and p30 protein plays a leading role in the internalization of the virus. The introduction of these proteins into an ASFV-infected cell culture to varying degrees affects the virus reproduction *in vitro* [10].

E. R. Tulman et al. (2009) showed the possibility of inhibiting ASF virus reproduction and delaying haemadsorption when using anti p54 and p30 recombinant proteins sera [6].

Another group of researchers has demonstrated that the introduction of a DNA vaccine, prepared on the basis of the plasmid DNA pCMV-PQ, carrying the p54 and p30 genes, induces the production of antibodies in mice [8].

As it was noted earlier, the use of p54 and p30 proteins in the considered experiments is not accidental, since they are structural proteins involved in viral penetration and internalization, although their role varies significantly.

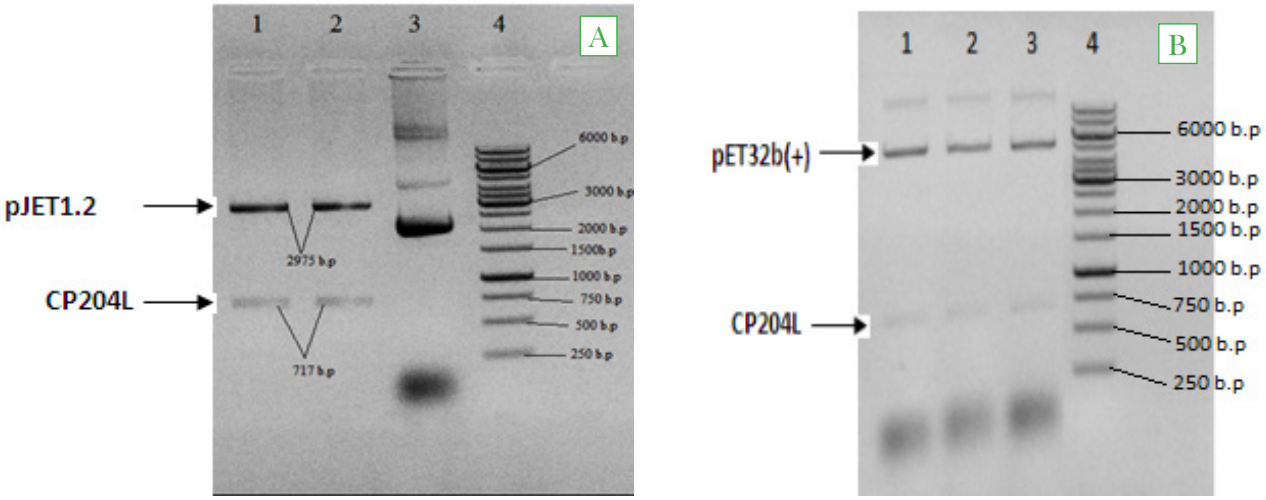


Fig. 1. Plasmid restriction analysis to determine the insert orientation and size

A (for pJET1.2-p30 plasmid): tracks 1 and 2 – pJET1.2-p30 electrophoresis after restriction; track 3 – recombinant pJET1.2/p30; track 4 – marker 1k (Thermo Fisher) DNA ladder with fragment length range of 10 000–250 b.p.;

B (for pET32b(+)/p30 plasmid): tracks 1, 2 and 3 – pET32b(+)-p30 after restriction; track 4 – marker 1k (Thermo Fisher).

p30 is one of the early viral proteins, which is encoded by the CP204L gene, has a molecular mass of 30 kDa and is one of the most immunogenic structural proteins responsible for the internalization of the ASF virus [4]. Expression of this protein, as a rule, begins 2–4 hours after infection and continues throughout the reproduction cycle. Thus, the presence of p30 synthesis indicates that the virus has penetrated into the cell, has lost its envelope, and the expression of the early CP204L gene has begun [7].

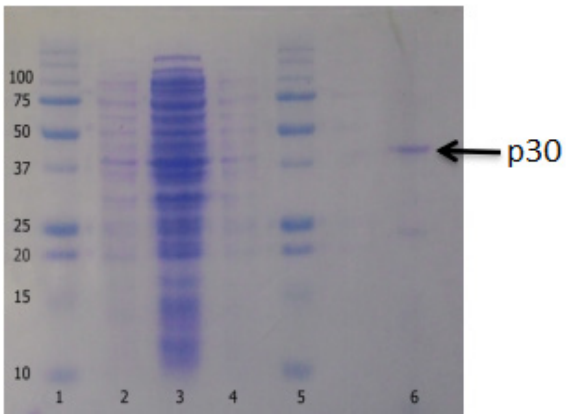
p54 is a protein encoded by the E183L gene, it is an antigenic structural protein responsible for the attachment and entry of the ASF virus, and has a molecular weight of 25–28 kDa [5].

It should be noted that vaccines based on recombinant proteins, modified viruses and DNA induce a cellular and humoral immune response to the ASF virus, but today their use does not provide 100% protection against infection. Obviously, further research is needed to determine protective proteins that can be included in the subunit or recombinant vaccine, as well as to establish the optimal immune mechanisms that need to be activated to provide reliable protection against ASF.

The aim of the study was to perform a comparative analysis of the effect of recombinant protein p30 and anti-p30 antibodies on ASF virus reproduction *in vitro*.

Fig. 2. Electropherogram in 10% PAGE indicating the efficiency of p30 purification using Ni-Sepharose

1 and 5 – markers;
2 and 4 – empty wells;
3 – cell lysate with p30;
6 – purified p30.



MATERIALS AND METHODS

The ASFV isolate Krasnodar 07/17 was used as a source of genomic DNA, it was isolated in July 2017 from a spleen sample from a dead domestic pig in the Krasnodar Krai.

The accumulation of the virus was carried out by passaging in the primary cell culture of porcine spleen (PSC) or porcine bone marrow (PBMC) for 2–3 passages.

The viral DNA was isolated from the virus suspension obtained after the 2nd or 3rd passage using DNA-Sorb-B kit (OOO "NextBio", Russia) in accordance with the manufacturer's instructions.

The design of the primers was carried out using the following web resources: for the analysis of the nucleotide sequences of the genome of the ASFV strains and isolates the international NCBI and EMBL databases were used; a comparative analysis of the homology of nucleotide sequences of genes was performed using BioEdit, version 7.2.5, and Benchling.

Amplification of ASFV CP204L gene was performed using polymerase chain reaction (PCR) with electrophoretic detection.

The gene amplification result was performed by electrophoretic separation in 1.0% agarose gel with detection in UV light after staining with ethidium bromide.

Cloning of the PCR product was performed in the non-expression vector pJET1.2/blunt using *Escherichia coli* cells JM-109 strain, according to the manufacturer's

Table 1
Indicators of optical density for different albumin concentrations

Albumin concentration, ng/µl	0	100	200	300	400	500	600	700	800	900	1000
Amount of saline solution, µl	1000	900	800	700	600	500	400	300	200	100	0
A280 value	0	0.069	0.109	0.183	0.244	0.274	0.371	0.418	0.491	0.543	0.617

instructions for the CloneJET PCR Cloning Kit (Thermo Fisher, USA).

PCR product or DNA restriction fragment was isolated from agarose gel using a kit of reagents for DNA elution from agarose gel (Qiagen, Germany) according to the manufacturer's instructions.

The CP204L gene was recloned into pET32b (+) expression vector using *E. coli* strain BL21 (DE3) pLysS competent cells (Promega, USA).

The preparation of p54 recombinant protein, as well as cloning of the PCR product was performed in non-expression and expression vectors using *E. coli* cells.

For a rapid assessment of the expression of the recombinant protein, an analysis of the whole-cell protein was performed using SDS-PAGE electrophoresis in 10% polyacrylamide gel according to U. K. Laemmli (1970) [9].

Purification of the recombinant protein was performed using Ni-Sepharose metal chelate chromatography (Sigma, USA).

Determination of total protein concentration was carried out according to Bradford method and using a diagram based on the determination of the absorption coefficient.

To study the effect of recombinant protein p30 on the reproduction of ASF virus *in vitro*, primary PBM cell culture and PS cell culture were used.

The level of ASFV reproduction in cell culture was assessed by haemadsorption and using the "ASF" test-system for ASFV detection by PCR (FBUN "Central Research Institute of Epidemiology" of Rospotrebnadzor, Russia) in accordance with the manufacturer's instructions.

RESULTS AND DISCUSSION

As a result of the studies, a PCR product was amplified, which included the CP204L full-length gene sequence. According to the results of sequencing carried out by N. G. Zinyakov (FGBI "ARRIAH"), the nucleotide composition of the obtained gene was 100% homologous with a similar sequence of the ASFV reference strain Georgia 2007/01 [3].

After cloning of the CP204L gene in pJET1.2/blunt, its orientation was determined by restriction analysis using XhoI and NcoI endonucleases. The size of the gene was established by electrophoretic separation in 1% agarose gel and the product was extracted from the gel for subsequent recloning into the pET32b(+) expression vector (Fig. 1).

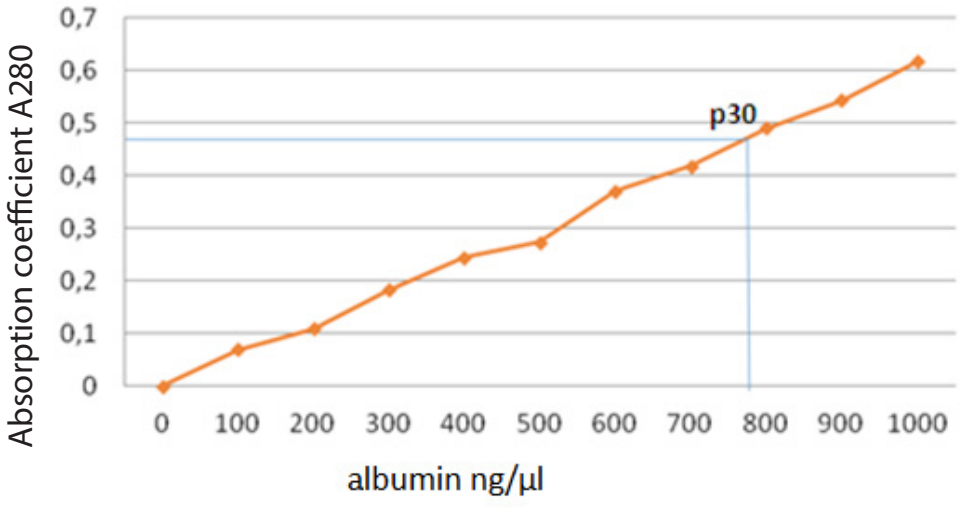
Efficiency of p30 purification based on Ni-Sepharose metal chelate chromatography was determined using SDS-PAGE electrophoresis in 10% polyacrylamide gel, in comparison to the initial cell lysate (Fig. 2).

The concentration of the purified protein was determined using a spectrophotometer based on dilution gradient of albumin in saline solution (Table 1, Fig. 3).

Thus, 3.2 ml of p30 with a concentration of 765 ng/µl was obtained as a result of protein purification.

Then, the nature of the effect of p30 on ASF virus reproduction *in vitro* was determined. For this purpose, cell culture growth medium was removed from the cell culture flasks with PS and PBM monolayer. Protein was added at 100, 200 and 300 ng per flask (T-25 volume of 25 cm^3) and then incubated in a shaker at 37 °C for 1 hour. Then the growth medium was added by 10 ml to each flask and the cell culture was infected with ASFV isolate Krasnodar 07/17 at the dose of 100 HAU per flask (~0.01 HAU per cell). Poludanum at the dose of 100 ng per flask was used to

Fig. 3. Determination of absorption coefficient in bovine serum albumin samples



control the impact on the ASF virus reproduction [1], and 100 ng of bovine serum albumin was added as a negative control. Each experiment was carried out in four variants: two flasks with PS cell culture and two flasks with PBM cell culture.

All samples were incubated for 7 days at 37 °C and the appearance of hemadsorption was registered using a light microscope. On day 3, 5 and 7 200 µl of culture fluid were taken from each flask for the study using hemadsorption test and real-time PCR. The test results are presented in table 2.

Average values of 4 samples are indicated in the table.

All values were calculated using the standard deviation formula:

$$SD = \sqrt{\frac{\sum(x_1 - \bar{x})^2}{n-1}}.$$

The results of the experiment on studying the effect of different amounts of p30 protein on the level of the virus reproduction *in vitro* are presented in the diagram constructed based on the virus titer values determined using the hemadsorption test and real-time PCR (Fig. 4).

The experiment results showed (Fig. 4) that addition of 100 ng of p30 and 100 ng of poludanum leads to an equivalent effect in changing the level of ASFV reproduction, that is, it significantly reduces its titer in the sample. The introduction of 200 ng of p30 led to a similar result by day 7 after infection. However, when a mixture containing 200 ng of p30 and 200 ng of p54 was introduced into the sample, the decrease in virus reproduction was more significant than when only 200 ng of p30 were used. The most significant effect on the virus reproduction was observed when adding 300 ng of p30, while complete inhibition of the virus reproduction was recorded throughout the entire experiment (7 days).

According to A. S. Kazakova (2012) a monospecific serum from rabbits immunized with recombinant p30 protein was used in similar experiments to study the ASFV reproduction *in vitro*. It was established by the author that antibodies of a monospecific rabbit serum against Rec p30e of the ASFV NVL-1 strain at a dilution of 1:8 delayed the appearance of hemadsorption for up to 5 days [2].

Table 2
Evaluation of effect of added protein on ASFV reproduction level by threshold cycle value (Ct)
n = 4

Protein/concentration	RT-PCR (Ct)		
	Day 3	Day 5	Day 7
p30/100 ng	22.2 ± 1.1	16.02 ± 0.31	13.36 ± 0.27
p30/200 ng	19.3 ± 0.5	16.13 ± 0.41	13.5 ± 0.15
p30/200 ng + p54/200 ng	27.8 ± 0.67	24.13 ± 0.59	19.8 ± 0.51
p30/300 ng	22.5 ± 0.31	22.38 ± 0.29	25.22 ± 0.57
poludanum/100 ng	21.9 ± 0.61	15.9 ± 0.44	13.14 ± 0.26
albumin/100 ng	18.4 ± 0.42	12.05 ± 0.6	10.03 ± 0.33
Cell culture control	–	–	–

The test results showed that the introduction of p30 had a greater impact on the reproduction of the ASF virus including complete inhibition, than the introduction of monospecific serum to p30 protein. These differences are explained by p30 direct blocking of the virus internalization stage, and consequently – its reproduction stage. When p30 protein is introduced into infected cell culture, the ASF virus and p30 protein compete for cellular receptors that are responsible for the internalization of the virus. As p30 protein blocks cell receptors, the stage of virus penetration is interrupted resulting in reduction or complete inhibition of the virus reproduction.

In its turn, the introduction of antibodies to p30 only partially blocks this stage of virus reproduction by binding virion protein and the protein synthesized again at an early stage. Due to the probable interaction with the p30 protein, antibodies to it have a less significant effect on the reproduction of the virus and only lead to a delay in the onset of hemadsorption.

Hence analysis of two different approaches to study the reproduction of ASFV *in vitro* confirmed the feasibility, priority and need to use them to study the characteristics of the virus reproduction.

CONCLUSION

As p30 protein plays an important role in the attachment and penetration of the ASFV into cells, it competes with viral proteins for receptors responsible for the penetration and intracellular transport of the virus when introduced into cell culture. Consequently it blocks the stage of internalization of the virus in the cell.

This phenomenon is demonstrated *in vitro* when recombinant p30 protein is introduced in cell cultures of porcine spleen and porcine bone marrow, while no change in virus's replication level is observed when adding albumin as a control.

Thus it was reliably established that ASFV replication decreases during the introduction of the recombinant protein p30 into cell cultures. It should be noted about the presence of a dose-dependent effect as the addition of 100 or 200 ng of p30 led to a significant decrease in the level of virus reproduction in the sample, and with the addition of 300 ng of p30, complete inhibition of virus's reproduction was observed during the entire period of experiment (7 days). It is remarkable that when a mixture of p30 and p54 was introduced into the sample, the reduc-

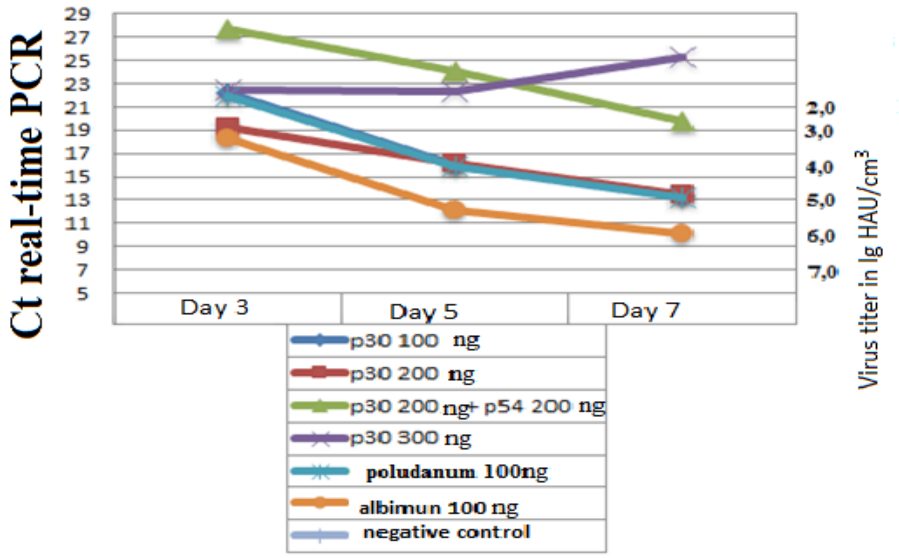


Fig. 4. Effect of ASFV p30 on virus reproduction in vitro

tion in reproduction was more significant than when only p30 was used.

It is evident that this method will contribute to determining the effect of other ASFV proteins with an unknown function on the reproduction of ASFV virus, which is necessary for identification of basic protective proteins and for establishment of optimal immune mechanisms providing reliable protection against ASF.

REFERENCES

1. Poludanum's influence on reproductive and respiratory syndrome, transmissible gastroenteritis and African swine fever viruses in primary and continuous cell cultures [Vliyanie poludana na reprodukciyu virusov reproduktivno-respiratornogo sindroma, transmissivnogo gastroehnterita i afrikanskoj chumy svinej v pervichnyh i perevivaemyh kul'turah kletok]. D. V. Sharypova, I. Yu. Zhukov, Ali Mazloum [et al.]. *Veterinary Science Today.* 2018; 1 (24): 37–41; DOI: 10.29326/2304-196X-2018-1-37-41 (in Russian).

2. Kazakova A. S. Construction of African swine fever virus recombinant proteins p72, p30 and p54 [Konstruirovanie producentov rekombinantnyh belkov r72, r30 i r54 virusa afrikanskoj chumy svinej]: Candidate Thesis (Biology). Pokrov; 2013 (in Russian).

3. Cloning of genes, coding for transmembrane proteins and proteins, responsible for African swine fever virus virulence [Klonirovanie genov, kodiruyushchih transmembrannye belki i belki, otvetstvennye za virulentnost' virusa afrikanskoj chumy svinej]. Ali Mazloum, N. G. Zinyakov, A. S. Igolkin, N. N. Vlasova. *Veterinary Science Today*. 2018; 2 (25): 3–7; DOI: 10.29326/2304-196X-2018-2-25-3-7(in Russian).

4. African swine fever virus controls the host transcription and cellular machinery of protein synthesis. E. G. Sánchez, A. Quintas, M. Nogal [et al.]. *Virus Res*. 2013; 173 (1): 58–75; DOI: 10.1016/j.virusres.2012.10.025.

5. African swine fever virus structural protein p54 is essential for the recruitment of envelope precursors to assembly sites. J. M. Rodríguez, R. García-Escudero, M. L. Salas, G. Andrés. *J. Virol*. 2004; 78 (8): 4299–4313; DOI: 10.1128/JVI.78.8.4299–4313.2004.

6. African swine fever virus. E. R. Tulman, G. A. Delhon, B. K. Ku, D. L. Rock. *Curr. Top. Microbiol. Immunol*. 2009; 328; 43–87; DOI: 10.1007/978-3-540-68618-7-2.

7. Correlation of cell surface marker expression with African swine fever virus infection. P. Lithgow, H. Takamatsu, D. Werling [et al.]. *Vet. Microbiol*. 2014; 168 (2–4): 413–419; DOI: 10.1016/j.vetmic.2013.12.001.

8. Enhancing DNA immunization by targeting ASFV antigens to SLA-II bearing cells. J. M. Argilaguet, E. Pérez-Martín, C. Gallardo [et al.]. *Vaccine*. 2011; 29 (33): 5379–5385; DOI: 10.1016/j.vaccine.2011.05.084.

9. Laemmli U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. 1970; 227 (5259): 680–685.

10. 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. P. Gómez-Puertas, F. Rodríguez, J. M. Oviedo [et al.]. *Virology*. 1998; 243 (2): 461–471; DOI: 10.1006/viro.1998.9068.

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