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TESTING OF COMBINED VACCINES AGAINST AUJESZKY'S DISEASE, PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME AND PORCINE PARVOVIRUS INFECTION FOR THEIR ANTIGENICITY

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SUMMARY

Results of cloning X69R, A179L, E248R, I215L and DP96R genes of ASF virus Krasnodar 07/17 isolate and analysis of their nucleotide sequences are presented. Obtained clones were added to the previously constructed clone library comprising clones of 8 genes of Krasnodar 06/12 isolate. Clones containing X69R, A179L, E248R, I215L and DP96R genes of ASF virus Krasnodar 07/17 isolate will be used for recombinant protein production and testing for their effect on *in vitro* virus reproduction and their role in the virus infectivity, level of clinical manifestations and virulence. Prokaryotic vector, pJET1.2/blunt, was used. Thus, the clone library available at the FGBI "ARRIAH" Reference Laboratory for African swine fever was supplemented by pJET1.2-X69R, pJET1.2-A179L, pJET1.2-E248R, pJET1.2-I215L and pJET1.2-DP96R plasmid constructions containing 5 genes of ASFV Krasnodar 07/17 isolate. Proportion of cloned virus genes was 3.01% of Krasnodar 07/17 isolate genome, hence, total amount of the clone library has reached 7.82%.

Key words: African swine fever (ASF), transmembrane proteins, proteins responsible for ASFV virulence, sequencing, gene clone library.

INTRODUCTION

Construction of representative DNA clone libraries is an efficient approach to the development of up-to-date diagnostic tools and recombinant vaccines. Moreover, use of clone libraries for the genetic analysis of highly dangerous disease agents allows to avoid the use of the infectious agent thus mitigating danger for the researcher.

The gene clone library is represented by the selection of recombinant plasmids (or bacterial cell clones comprising them) bearing different genes of specific host. The first gene clone library was constructed by F. Sanger et al. in 1977. [11, 12].

In 2002, S. D. Kollnberger et al. screened the library of expressing genes of African swine fever virus (ASFV) using polyclonal antisera (from pigs recovered from the infection with virulent ASFV) and identified 14 serological immunodeterminants including non-structural proteins (pB602L, pC44L, pCP312R, pE183L, pK145R and pK205R), structural proteins (pA104R, p10/pK78R, p30/pCP204L, p54/pE183L, p72/pB646L) and the following viral enzymes: ribonucleotide reductase (Fp334L, pF778R), DNA ligase (pNP419L) and thymidine kinase (pK169R) [9].

In 2014, in the Russian Federation the first library of clones of CP204L, KP177R, O61R, EP402R, CP530R, E183L genes and B646L CP2475L gene fragment of ASFV Krasnodar 06/12 isolate was constructed. The genes and gene fragments were cloned within pJET 1.2. vector molecule. The proportion of cloned ASFV genes amounted to 4.81% of Krasnodar 06/12 isolate genome [2]. This library did not include X69R, A179L, E248R, I215L and DP96R genes, coding pX69R, pA179L, pE248R, ubiquitin-conjugating enzyme (E2) and UK proteins, respectively. These genes play an important role in the maintenance of ASF agent virulence and effect the degree of disease clinical manifestation.

Protein pE248R coded by gene E248R located in the left terminal variable region of the virus genome was demonstrated to be essential for the formation of viable infectious ASFV virions. It is specified by the properties similar to p54 protein and it is localized on the inner lipoprotein membrane of the virus particle [5].

Protein pA179L/5HL or p21 (viral homologue of Bcl2 (vBcl2)) is coded by A179L gene located in the cen-

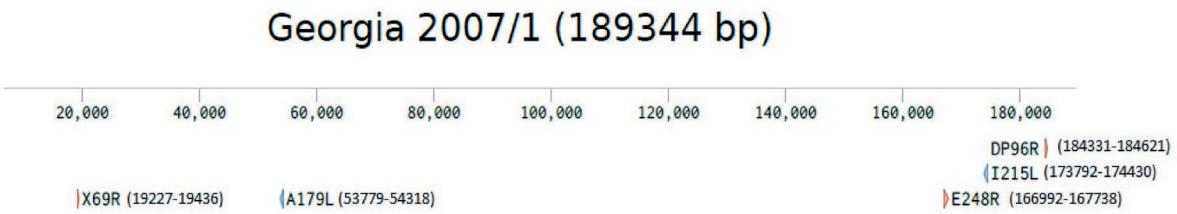


Fig. 1. Layout of full-size genome of ASFV Georgia 2007/1 strain with location of ORF of X69R, A179L, E248R, I215L and DP96R genes

The arrows designate the direction of the gene open reading frame; the location is designated in brackets. Upstream genes (L) are designated in blue; downstream genes are designated in red (R).

tral conserved region of the ASFV genome. It has molecular weight of 19 kDa and blocks apoptosis of infected cells [6, 10]. The function of this protein was demonstrated during the expression in heterologous systems such as vaccinia virus or baculovirus [4]. This protein is synthesized both at the early and late stages of the infection thus supporting its critical role in the cell survival at different stages of the ASFV life cycle.

UK protein of 10.7 kDa molecular weight consists of 96 amino acids and is coded by DP96R gene located at the right variable terminus of the genome. Analysis of DP96R nucleotide sequences deposited in gene databases did not demonstrate any significant similarity between DP96R and other genes of known viruses. It is well known that this gene is responsible for ASFV virulence and degree of clinical manifestation. All experimental animals recovered following the infection with genetically modified DP96R-free virus [3].

Ubiquitin-conjugating enzyme (E2) coded by I215L gene is homologous to the protein family that bind ubiquitin in the infected cell [15]. The process starts with ubiquitin binding with the proteins for its further modification: three proteins (E1, E2 and E3) are involved in the process. Enzyme E2 interacts with enzyme E1 and one or several E3 enzymes for their activity regulation; it can also interact directly with the proteins and play its role in determination of their ubiquitin-mediated modification route [7].

69-amino-acid pX69R protein is coded by a currently non-described X69R genome. However, protein antigenicity prognostic analysis using Vaxign system demonstrated that X69R codes the trans-membrane structural protein having an ability to effectively induce the antibody production. Assessment of the ASFV protein antigenicity level classified the virus as only the 22nd in the line of candidates to be used for the vaccine design [8, 16, 17]. Therefore, the clone library including copies of these genes can be used for the development of recombinant and DNA-vaccines against ASF or for the production of genetically modified virus. This fact determined the orientation of the works to the replenishment of the representative ASFV gene clone library.

The work was also aimed at the production of recombinant plasmids bearing full-size genes of transboundary proteins and proteins responsible for African swine fever virus virulence.

MATERIALS AND METHODS

ASFV Krasnodar 07/17 isolate recovered from pig spleen sample (July 2017, Krasnodar Krai, OOO APK Petrovsky) was selected for cloning. The pathological material was used for the infection of the pig spleen cell culture. The second passage virus-containing suspension was used for DNA extraction.

The viral DNA was extracted using DNA-sorb-B test-kit (FBUN Central Research Institute of Epidemiology, Rospotrebnadzor, Russia) according to the manufacturer's instruction [1].

Polymerase chain reaction (PCR) results were read using 1.0% agarose gel electrophoresis with subsequent UV detection following staining with ethidium bromide. The fragment length was determined using 1kb DNA ladder (Thermo Fisher) for the range of 250-10000 bp.

For sequencing, the PCR-products were extracted from the agarose gel using gel extraction test-kit (Qiagen) according to the manufacturer's instructions [14].

The PCR-products were cloned according to CloneJET PCR Cloning Kit (Thermo Fisher) manual using pJET1.2/blunt cloning vector and *E. coli* JM-109 strain [13].

The obtained amplicons were sequenced using BigDye Terminator Cycle Sequencing kit with ABIPrism 3100 Genetic Analyzer (DNA-sequencer) (Applied Biosystems, USA).

Genome nucleotide sequences of ASFV strains and isolates were analyzed using data from international databases such as NCBI and EMBL. BioEdit version 7.2.5 and Benchling software were used for the comparison of the nucleotide sequence homology.

RESULTS AND DISCUSSION

Cloning of ASFV genes and analysis of their nucleotide sequences included several stages:

1. Primer design

Optimal sites for primer design were defined basing on the publications and analysis of X69R, A179L, E248R, I215L and DP96R on the Georgia 2007/1 ASFV genome physical map (Fig. 1).

Using Benchling software five primer pairs that flanked the above listed full-size genes were calculated (see the Table).

2. PCR optimization

Primer annealing temperature was adjusted using Mastercycler-nexus amplifier (Eppendorf, Germany) at 42°-62°C gradient range with 1°C increase. The experiments resulted in optimization of conditions of the virus genome fragments' amplification for their further cloning. The optimal annealing temperatures are established to be within 50–55°C.

3. Cloning of ASFV genes in pJET1.2 blunt vector

The amplified full-size gene copies (Fig. 2) were used for construction of prokaryotic pJET1.2-vectored recombinant

Table
Primers used for ASFV gene amplification

Gene name	Position in the genome	ORF direction	Primers	Primers' positions	Fragment size (bp)
E248R	166992–167738	From left to right	E248R-F	166983 167008	759
			E248R-R	167721 167742	
I215L	173792–174430	From right to left	I215L-F	173754 173778	717
			I215L-R	174449 174471	
DP96R	184331–184621	From left to right	DP96R-F	184314 184334	404
			DP96R-R	183698 184718	
A179L	53779–54318	From right to left	A179L-U	53748 53767	679
			A179L-D	54408 54427	
X69R	19227–19436	From left to right	X69R-F	18470 18493	1140
			X69R-R	19586 19610	

plasmids with subsequent *E. coli* transfection with the produced constructions.

Vector-containing insertions were ligated by blunt ends. Averagely, 50-100 ng of DNA and 25 ng/ml of pJET1.2/blunt vector were used for ligation of PCR products of over 1000 bp; in case of PCR products of below 1000 bp – 20-50 ng/ml of DNA and 25 ng/ml of pJET1.2/blunt vector were used.

For transformation competent cells of *E. coli* JM109 strain were used at 10^6/50µl.

Prokaryotic vector pJET1.2/blunt was chosen for PCR-product cloning due to the convenience and simplicity of its handling, as this method does not require PCR-product purification from the reaction mixture components. Use of this vector furthermore allows cloning with 100% positive clone availability thus avoiding colony screening.

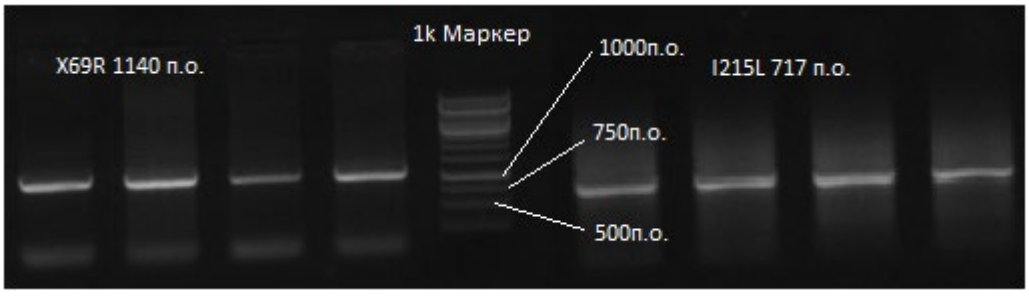
Plasmid DNA of the produced clones was PCR tested for insertions and was used for further sequencing. For this purpose, three clones bearing homologous insertion of each gene were selected. Analysis of the nucleotide sequences of the cloned PCR-products confirmed that full-size genes were cloned in pJET1.2-blunt plasmid.

4. Nucleotide sequencing of ASFV genome

Since determining the gene orientation within the cloning plasmid is an essential requirement for its subsequent recloning into expressing plasmids, primers flanking the polylinker-region of pJET1.2-blunt plasmid were used during sequencing. This allowed determination of the cloned gene orientation. The analysis results demonstrated that A179L and X69R genes were cloned in reverse orientation and E248R, I215L and DP96R genes – in direct orientation.

Fig. 2. Results of PCR-product electrophoretic separation in 1% agarose gel (ASFV Krasnodar 07/17 isolate)

To the left of the marker – PCR-product obtained using primers for amplification of genome fragments containing X69R gene; to the right of the marker – fragments containing I215L gene.



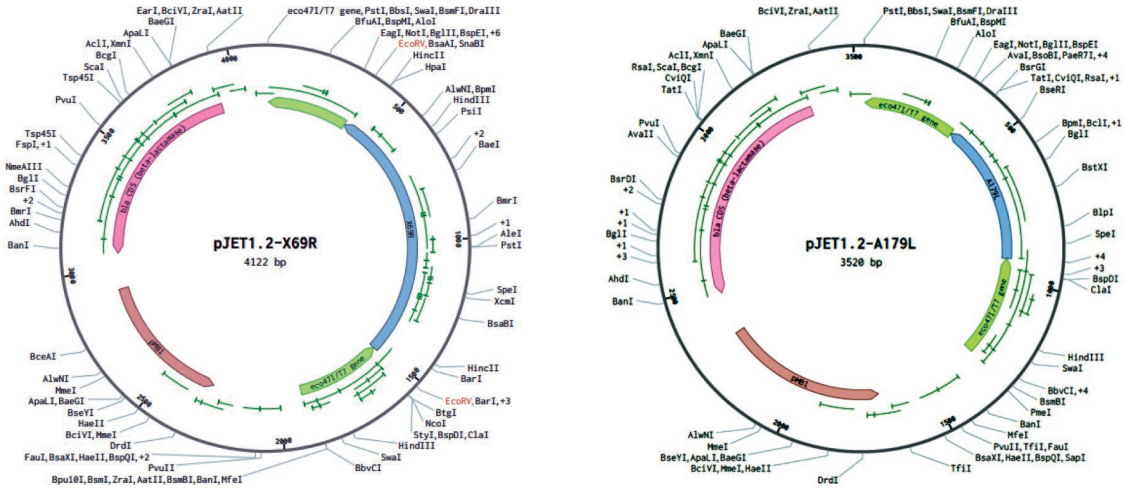


Fig. 3. Diagram of recombinant pJET1.2 plasmid bearing ASFV genes X69R (left) and A179L (right)

Blue arrow – cloned gene insertion; green arrow – pJET1.2 plasmid polylinker; pink arrow – β-lactamase gene. The recombinant plasmid name and size are in the center.

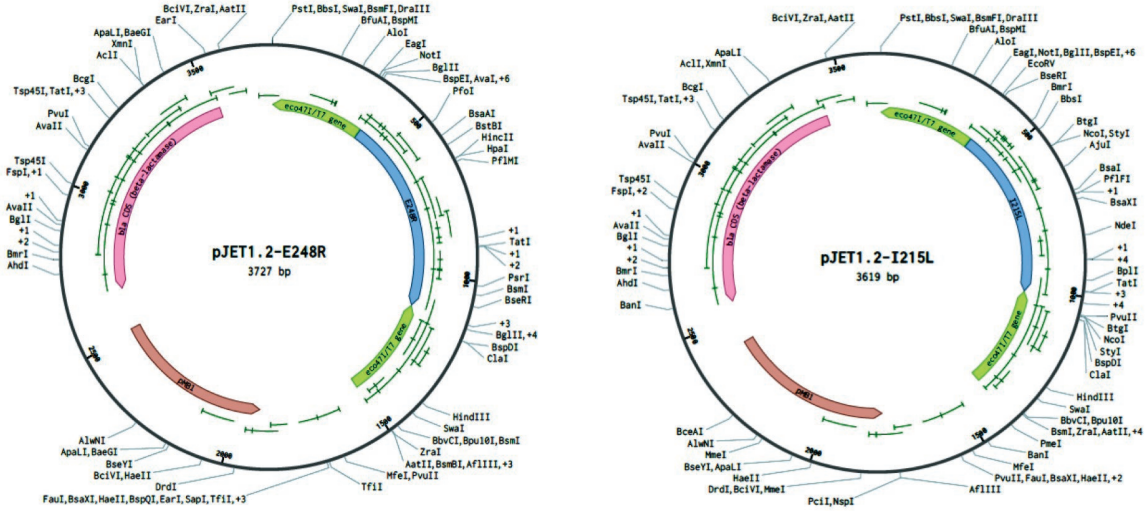


Fig. 4. Diagram of recombinant pJET1.2 plasmid bearing ASFV genes E248R (left) and I215L (right)

Blue arrow – cloned gene insertion; green arrow – pJET1.2 plasmid polylinker; pink arrow – β-lactamase gene. The recombinant plasmid name and size are in the center.

Full-size nucleotide sequences of X69R, A179L, E248R, I215L and DP96R genes of Krasnodar 07/17 isolate were also analyzed. The results demonstrated that they were identical to the similar sequences of Georgia 2007/1 isolate.

Thus as a result of the work performed five ASFV genes were produced in the recombinant plasmids: pJET1.2-X69R bearing 1140 bp insertion, and pJET1.2-A179L bearing 679 bp insertion (Fig. 3); pJET1.2-E248R bearing 759 bp insertion and pJET1.2-I215L bearing 717 bp insertion (Fig. 4); pJET1.2-DP96R bearing 404 bp insertion (Fig. 5).

The obtained clones were added to the clone library containing 8 genes coding immunologically relevant ASFV proteins (B646L (p72), CP204L (p30), KP177R (p22), O61R (p12), EP402R (CD2v), CP530R (pp62), CP2475L (pp220) and E183L (p54)) and established in 2014 by A. A. Varentsova, et al. [2]. The portion of cloned ASFV genes amounted to 3.01% of the genome of Krasnodar 07/17 isolate; thus overall volume of the library reached 7.82%.

The ASFV genes described in the paper were selected from two groups: transmembrane protein-coding genes (X69R and E248R), and genes (A179L, DP96R and I215L) coding proteins affecting ASF virus virulence and degree of clinical manifestation.

The clone library of the first group of the genes is intended for the recloning to expressing plasmids and production of recombinant proteins for examination of their effect on the virus reproduction and for determination of their role in this process. The clone library of the second group of the genes can be used for production of genetically modified virus and its comparison analysis thus being the first stage of the ASFV vaccine development.

CONCLUSION

The selected oligonucleotide primers allowed for amplification of the ASFV full-size genes X69R, A179L, E248R, I215L and DP96R that code transmembrane proteins and

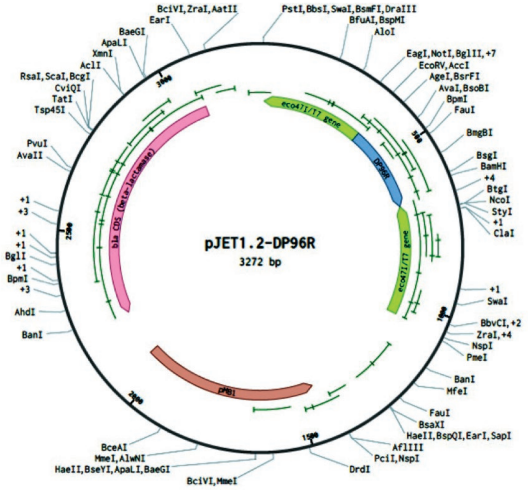


Fig. 5. Diagram of recombinant pJET1.2 plasmid bearing ASFV gene DP96R

Blue arrow – cloned gene insertion;
green arrow – pJET1.2 plasmid polylinker;
pink arrow – β-lactamase gene.
The recombinant plasmid name and size are in the center.

proteins affecting the virus virulence and for determination of their nucleotide sequences for Krasnodar 07/17 isolate.

Recombinant plasmid constructions bearing amplified full-size genes were produced.

Plasmid constructions pJET1.2-X69R, pJET1.2-A179L, pJET1.2-E248R, pJET1.2-I215L and pJET1.2-DP96R included in the above described clone library will be used for production of recombinant proteins and examination of their effect on ASFV reproduction *in vitro*. Furthermore, they can be used for determination of specific structural properties of individual genes, development of alternative sources of the genetic material, production of recombinant viruses and DNA vaccines against ASF and as positive controls in PCR-diagnosis.

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