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OBTAINING A STABLE CELL LINE EXPRESSING RECOMBINANT I329L PROTEIN OF AFRICAN SWINE FEVER VIRUS

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Abstract

The African swine fever virus (ASFV), a large DNA virus with icosahedral morphology, is the only representative of the family *Asfarviridae*. ASFV has a wide list of mechanisms for evading the host's immune system. This fact hinders the development the vaccines against ASF. One of the approaches used by the virus in immune evasion is the mimicry of Toll-like receptors (TLR) by immunomodulating proteins. ASFV immunomodulatory proteins are the most valuable tools for the understanding of the pathogenesis of the disease and to create a means of combating the disease. One such ASFV protein is pI329L, the antagonist and TLR3 signaling inhibitor, which reduces the interferon response of the body. Protein pI329L inhibits TLR3-mediated activation of NF-κB and induction of INF-b through the activation of TLR3 with its ligand — viral DNA, RNA and poly (I:C). Removing this protein from ASFV particles is a rational approach to developing a weakened virus vaccine. Therefore, I329L is characterized as a viral TLR3 antagonist, which negatively affects interferon antiviral response of the host. Purpose of the work was to obtain a CHO cell line stably expressing a TLR3 antagonist, the recombinant I329L protein of ASFV. Here, we designed the plasmid pBMN-I329-his, carrying the full-length I329L gene with 6xHis-tag at the C-terminus. By electroporation with plasmid pBMN-I329-his of the CHO cell line and further stabilization on a selective antibiotic (5 μg/ml puromycin), a stable CHO-I329L-His cell line was derived. The insertion of the I329L gene into the genome of the cell was confirmed by PCR using primers of the specific gene, followed by nucleotide sequencing, using as the template DNA isolated from the cells CHO-I329L-His. Western Blot confirmed the presence of I329L protein in the cell lysates of CHO-I329L-His. As a result of the analysis it was established that the size of the recombinant protein was 55 kDa compared to calculated 35 kDa. The sequential deglycosylation of endoglycosidases PNGase and EndoH of the target protein resulted in an increase in its electrophoretic mobility and detection of specific bands of ~ 37 and ~ 35 kDa. This fact confirms the high degree of glycosylation of the target molecule, which leads to a lower electrophoretic mobility. Additionally, the recombinant I329L protein was recognized by hyperimmune sera against the ASFV, which indicated its authenticity. The obtained stable cell line CHO-I329L-His is deposited in the cell culture museum of Federal Research Center for Virology and Microbiology and can be used to study the mechanisms of action of immunomodulating proteins, such as pI329L of the ASFV, and, therefore, to get deeper insight of the African swine fever virus biology.

Keywords: African swine fever, ASFV, TLR3 signalling, protein expression, recombinant pI329L, stable cell line

African swine fever (ASF; the causative agent is African swine fever virus, ASFV, *Asfivirus*, *Asfarviridae*) is infectious disease of domestic and wild pigs, characterized by high mortality and contagiousness, which occurs in hyper-

acute, acute, subacute and chronic forms and can be transmitted from sick and infected animals through contacts and by alimentary ways. In the Russian Federation, the ASFV has registered since 2007. Over recent years, the country has considered as an area of disease risk [1].

The virus genome consists of linear double-stranded DNA (dsDNA) and is between approximately 170 and 190 kbp in size, depending on the isolate. All members of the pig family *Suidae* are susceptible to ASFV infection. The main virus reservoir is ticks *Ornithodoros* [2]. The genome nucleotide sequence of reference ASFV strain Ba71V contains 150 open reading frames (ORFs). In ASFV infected cells, 95 polypeptides with a molecular weight from 10 to 220 kDa were detected [1]. ASFV has various mechanisms for evading the host's immune system. It becomes the main hindrance for development the vaccines against disease [3]. One way of immune evasion is through mimicry of Toll-like receptors (TLR). These receptors are responsible for viral double-stranded RNA (DNA) recognition and activate innate immunity [4]. Nowadays, 13 mammalian Toll-like receptors are known. They are activated by various ligands, basically by bacterial and viral components. The receptors differ in adaptor peptides with which their cytosolic fragments are linked [5].

One of the ASFV proteins involved in the modulation by the mediated Toll-like receptor of host defense, is pI329L, the TLR3 antagonist and signaling inhibitor [6, 7]. The functional analysis indicates that pI329L inhibits TLR3-mediated activation of NF-κB and IFN- b through the TLR3 activation with ligand, i.e. the viral DNA, RNA or poly (I:C) [8, 9]. So, the I329L protein serves as a viral TLR3 antogonist that negatively impacts on the interferon antiviral host response [10]. Nowadays, there is no information about the I329L protein of ASFV which is circulating in Russia. There is no data on its genetic stability [11, 12], and also no tools (e.g. stable cell line) for studying mechanism of immunomodulating activity of pI329L protein of Russian ASFV isolates.

In this paper, we for the first time reported data on the recombinant I329L protein authentic to viral ASFV protein, which was produced in Chinese hamster ovary (CHO) cell lines.

The aim of the research is obtaining a stable cell line to express recombinant I329L protein of the African swine fever virus.

Technique. The nucleotide and amino acid sequences of I329L protein was taken from the UniProt database (reference number E0WM90). pBMN-I329L-his plasmid was designed in Clone Manager 7.0 software (Sci-Ed Software, USA). DNA was isolated from the reference strain Stavropol 01/08 ASFV (State Collection of FRCV&M) [20] using the QIAamp DNA Blood Kit and Mini Kit (Qiagen N.V., Germany).

PCR was performed using Phusion polymerase (NEB, USA) according to the manufacturer's instructions. The reaction mixture consisted of 10 μl of 5× Phusion HF buffer, 1 μl of 10 mM dNTPs, 2 μl of 10 μM F329his, 2 μl of 10 μm R329his, 1.5 μl of DMSO, 0.5 μl of Phusion DNA Polymerase, 1 μl of viral DNA and 31 μl of sterile water. A pair of primers used was F329his (forward) 5′-TATATAAAGCTTGCCACCATGCTAAGGGTTTTCATA-3′, R329his (reversed) 5′-TATATATCTAGATTATCAATGGTGGTGGTGATGGTGCTTTCTTCTTA-CACGAGA-3′. R329his containes a His-tag peptide at the C-terminus. Amplification of the target gene was carried out according to the protocol: pre-denaturation for 5 min at 98 °C; denaturation for 30 sec at 98 °C, primer annealing for 30 sec at 52 °C, elongation for 60 sec at 72 °C (25 cycles). An amplified product of the expected size was identified in 1.3 % TBE-agarose gel with ethidium bromide.

DNA was purified from the agarose gel using the Gel Extraction Kit

(Qiagen N.V., Germany) according to the manufacturer's instructions. The PCR product of *I329L* was cloned at HindIII and XbaI restriction sites (NEB, USA) into the pBMN plasmid vector (Addgene, USA), having ampicillin and puromycin resistance genes. Ligation and insertion were performed at a room temperature for 1 hour using T4 DNA ligase (NEB, USA) in a volume of 5 μl (0.5 μl of 10× ligase buffer, 1 μl of the linearized pBMN vector, 0.5 μl of *I329L* PCR product, 0.5 μl of T4 DNA ligase and 2.5 μl of sterile water).

Transforming of *Escherichia coli* XL-10 Gold strain, genotype endA1 glnV44 recA1 thi-1 gyrA96 relA1 lac Hte Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 tetR F′(proAB lacIqZΔM15 Tn10(TetR Amy CmR), was made using ligated mixture by standard heat shock method. Plasmid DNA was isolated with Plasmid Mini Kit (Qiagen NV, Germany) The isolated plasmid DNA of clones were tested by analytical restriction with the use of flanking endonucleases HindIII and XbaI. The reproduction accuracy of the cloned fragment was confirmed by sequencing on a genetic analyzer Applied Biosystems 3130xl (Applied Biosystems, USA).

Transfection of the CHO cell line (Chinese hamster ovary cells) was performed by electroporation on a Gene Pulse Xcell device (Bio-Rad, USA). The continuous cell line CHO was incubated in a shaker at 37 °C and 5 % CO_2 in BalanCD medium (Irvine, USA) with 500,000 units of penicillin, 100 μg/ml of streptomycin, and 4 mM L-glutamine. Viability and the amount of living cells were evaluated by staining with 1 % trypan blue solution (Gibco, USA). For this, to a vial containing 20 μl of the cell suspension, 80 μl of 1 % trypan blue solution which stains dead cells was added and shaken vigorously. The results were integrated on an automatic cell counter NucleoCounter NC100 (ChemoMetec, Denmark).

Protein I329L of ASFV was isolated using magnetic particles DynaBeads His-tag (Thermo Scientific, USA). Peptide N-Glycosidase F (PNGase F) (NEB, USA) and endoglycosidase H (EndoH) (NEB, USA) were used for I329L protein deglycosylation. Detection of the target protein was performed by western blotting in a 12.5 % polyacrylamide gel, followed by transfer to a Trans-Blot Turbo system (Bio-Rad, USA) on a nitrocellulose membrane (Bio-Rad, USA). The membrane was incubated with rabbit polyclonal antibodies to 6× His-tag (HRP) (Abcam, USA). The chemiluminescent reagents were used to visualize the reaction (Advansta, USA). The specificity of the recombinant I329L protein was recognized in reaction with hyperimmune swine antisera against the Stavropol 01/08 ASFV (State Collection of FRCV&M) from infected animals, and with commercial species-specific goat antibodies against pig IgG, conjugated with horsradish peroxidase (HRP) (Santa Cruz Biotechnology, USA).

Results. In this research, we used DNA isolated from culture of pig bone marrow cells infected with Stavropol strain 01/08 ASFV. The resulting matrix was used in PCR with gene specific primers (F329his and R329his) to amplifyfragment with restriction sites for subsequent cloning into the pBMN vector. A resultant PCR product of 1023 bp was obtained. In ligation, the ratio of the vector and the insert was 1:3.

Analytical restriction at flanking endonuclease sites confirmed the presence of a specific insert of *I329L* gene (Fig. 1, B) in four clones. They were verified by sequencing for the insert accuracy and the presence of substitutions or deletions. A comparative alignment of nucleotide and amino acid sequences selected the pBMN-I329L-his clone, identical in its sequence to the reference gene *I329L* of strain Georgia/wb/2007 ASFV (FR682468).

To obtain stable CHO-I329L-His cell line, a culture of CHO cells in the phase of logarithmic growth was used. The cells viability after transfection with

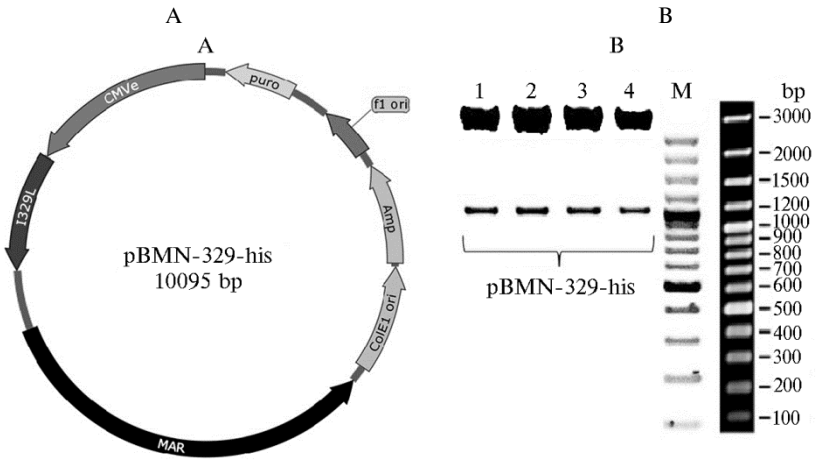

Fig. 1. Map of pBMN-I329L-his plasmid caring the target *I329L* gene of African swine fever virus (ASFV) (A) **and screening the plasmid pBMN-I329L-his clones by analytical restriction using HindIII and XbaI** (NEB, USA) (B)**:** 1-4 — clones Nos. 1-4 pBMN-I329L-his, M - molecular weight marker 100 bp Plus (Fermentas, USA).

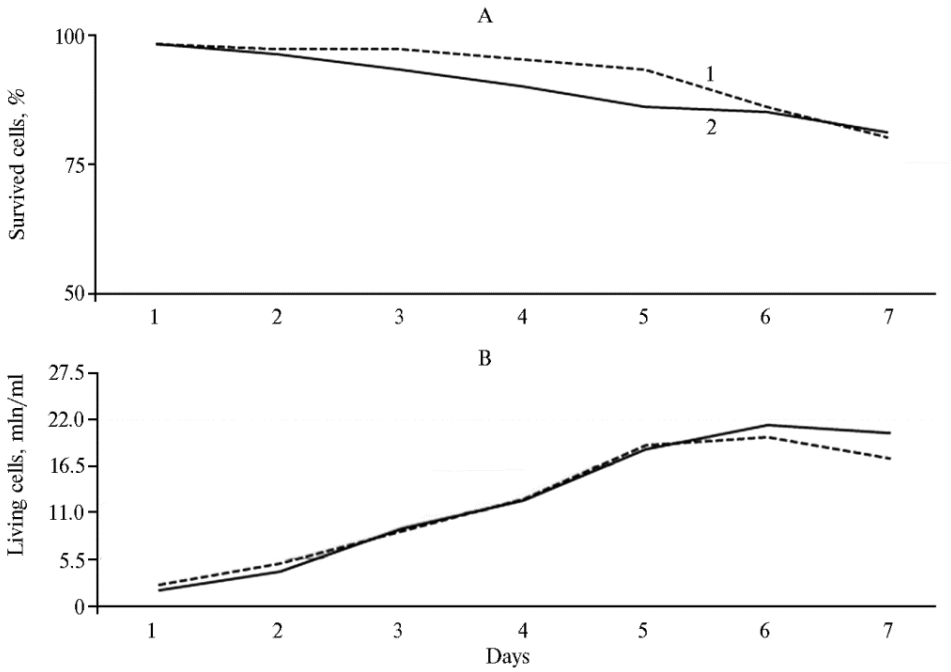

Fig. 2. Vitality (A) **and growth rate (B) of the CHO-I329L-His cell line** (1) **and the original CHO cell line** (2) **during 7-day culture in BalanCD medium supplemented with 5 μg/ml puromycin** (Appli-Chem, Germany).

pBMN-I329L-his plasmid was tested in 24 and in 48 hours with a trypan blue. Twenty-four hours after CHO cells transfection with pBMN-I329L-his plasmid, cells viability was 70 %, the counts of the living cell suspension was 1.0 million/ml; in the control transfection without plasmid, these values were 92 % and 1.0 million/ml, respectively. Analysis performed repeatedly 48 hour after transfection showed that the proportion of living cells increased up to 83 %, and the amount of living and transfected plasmid cells were up to 2.2 million/ml; in control, the figures increased to 94 % and 4.0 million/ml, respectively. The obtained data confirmed the efficiency of CHO cell transfection and the possibility of further selection of transfected cells in a puromycin containing medium (Ap-

pliChem, Germany). Forty-eight hours after transfection, the overall cells pool was transferred to a BalanCD growth medium with puromycin at 5 μg/ml final concentration.

We evaluated the dynamics of viability and the counts of viable transfected cells in comparison to the parent CHO cell line (Fig. 2) in model 7-day culture of suspension. The results showed that the 80 % threshold of viability was reached only on day 7 of culture. Over 7 days, in the stable CHO-I329L-His cell line, there were more than 80 % of viable cells.

ASFV *I329L* gene integration into CHO cell genome was confirmed by PCR with the reference primers for b-actin (NM_001244575.1) and cytochrome b (AB033693) of CHO cell line as an internal positive control. As a result, the *I329L* gene sequences were detected in total genomic DNA isolated from the stable CHO-I329L-His cell line (Fig. 3, A).

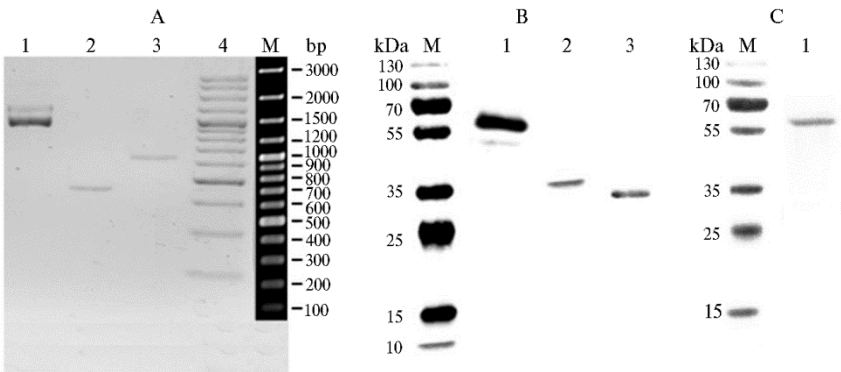

Fig. 3. Analysis of ASFV gene *I329L* integration and CHO-I329L-His cell line by PCR and immunobloting.

A: Detection of the *I329L* gene in CHO-I329L-His cells by electrophoretic separation of PCR products: 1 — amplified *I329L* gene from stable cell line CHO-I329L-His; 2 — amplified b-actin gene from stable cell line CHO-I329L-His; 3 — amplified cytochrome b gene from stable cell line CHO-I329L-His; M is molecular weight marker 100 bp Plus (Fermentas, USA).

B: Immonoblotting of ASFV recombinant protein I329L with His-taq antibodies after deglycosylation by endoglycosidases EndoH and PNGaseF: 1 — protein I329L isolated on magnetic particles DynaBeads His-tag (Thermo Scientific, USA); 2 — protein I329L cleaved with endoglycosidase EndoH (NEB, USA); 3 — protein I329L cleaved with endoglycosidase PNGase F (NEB, USA); M is the molecular weight marker Page Presteined Rule (Fermentas, USA).

C: Immonoblotting of recombinant protein I329L bound to hyperimmune serum antibodies against ASFV: 1 — I329L protein isolated on DynaBeads His-tag magnetic particles (Thermo Scientific, USA); M is molecular weight marker Page Presteined Ruler (Fermentas, USA).

Analysis of the target ASFV I329L protein expression was performed by western blotting with specific antibodies to His-tag located at C-terminus of the molecule. To do so, cells (5×10) selected from the continuous cell line were lysed, followed by concentration with DynaBeads His-Tag magnetic particles. Western blot confirmed the presence of protein I329L in the stable CHO-I329L-His cell line. However, the expressed recombinant I329L protein of 55-60 kDa in size (see Fig. 3, B) did not match to expected molecular weight of 35 kDa. This fact can be explained by the higher glycosylation of I329L protein, which reduces electrophoretic mobility of the molecule. These results are also confirmed by bioinformatic analysis which showed 9 N-glycosylation sites [15, 17[. An additional weak band of 35 kDa (see Fig. 3, B) could be explained by the protein translation from one of the three start codons (ATG) in its extracellular domain [17, 18]. It should be noted that the electrophoregram also showed traces of the target protein proteolysis.

As a result of treatment of the recombinant protein I329L with endogly-

cosidases, its molecular weight decreased to ~37 kDa for EndoH and ~35 kDa for PNGaseF, so originally, the expressed protein I329L was highly glycosylated (see Fig. 3, B). Besides, the molecular weight of I329L treated with PNGaseF was lower than that after EndoH treatment, indicating the presence of a small amount of complex glycans.

In immunoblotting test of binding I329L with hyperimmune serum of animals infected with strain Stavropol 01/08, the target protein I329L formed immune complexes with polyclonal antibodies to ASFV (see Fig. 3, C).

Thus, we developed stable CHO-I329L-His cell line which express the recombinant full-length transmembrane I329L protein of the African swine fever virus ASFV. The CHO-I329L-His cell line has similar phenotypic and growth properties with the parent CHO line. The ASFV recombinant protein I329L is non-toxic for CHO cells. The insertion of the *I329L* gene into genome of CHO cells was confirmed by PCR and by analysis of the target protein expression. The presence of glycosylated forms of protein I329L was revealed. Binding I329L with hyperimmune serum antibodies against ASFV is indicative of its antigen specificity. The CHO-I329L-His cell line is a unique model to study the ASFV interaction with a cell and to develop candidate vaccines based on defective recombinant ASFV. Stable cell line CHO-I329L-His was deposited in the Cell Culture Museum of the Federal Research Center for Virology and Microbiology. The cell culture can be used in studying mechanisms of immunomodulating protein activity and will provide new data on ASFV biology.

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