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RESEARCH ARTICLE

Production of the recombinant hemagglutinin protein of the swine influenza virus A/H1N1 and analysis of its physicochemical and antigenic properties

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Abstract

Objectives. To analyze the physicochemical and antigenic properties of recombinant hemagglutinin protein of swine influenza virus strain A/H1N1 (swH1-His) obtained by transduction of suspension line HEK293 with recombinant human adenovirus serotype 5.

Methods. The *de novo* assembly of the target hemagglutinin gene was performed via the polymerase chain reaction. Recombinant adenovirus recAd5-swH1-His was obtained using the AdEasy™ Adenoviral Vector System kit. Accumulation of preparative amounts of recombinant protein was performed by transduction of recAd5-swH1-His suspension culture of HEK293 cells in a wave-type bioreactor. Recombinant hemagglutinin was isolated from the culture medium by metal-chelate affinity purification on a sorbent. The actual molecular mass and its correspondence to the expected value, as well as the presence of histidine residues were shown by electrophoresis and Western blot. The antigenic specificity of swH1-His was determined by indirect enzyme-linked immunosorbent assay with specific sera.

Results. Recombinant hemagglutinin swH1-His was obtained in the amount of 1.2 mg from 50 mL of culture fluid. The compliance of its mass with the declared molecular mass (≈70 kDa) was confirmed along with the presence of cross-linking with histidine residues. The antigenic specificity of swH1-His in reaction with sera was demonstrated.

Conclusions. The physicochemical and antigenic characteristics of recombinant protein hemagglutinin of swine influenza A/H1N1 (swH1-His) obtained by transduction of HEK293 cells with recombinant human adenovirus of serotype 5 were determined. The obtained recombinant hemagglutinin can be used as an antigen for animal and human influenza diagnostic purposes.

Keywords

recombinant protein, recombinant human serotype 5 virus, purification of recombinant protein, antigenic specificity, enzyme-linked immunosorbent assay

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НАУЧНАЯ СТАТЬЯ

Получение и анализ физико-химических и антигенных свойств рекомбинантного белка геммагглютинина вируса свиного гриппа A/H1N1

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Аннотация

Цели. Анализ физико-химических и антигенных свойств рекомбинантного белка гемагглютинина вируса свиного гриппа штамма A/H1N1 — swH1-His — полученного путем трансдукции суспензионной линии HEK293 рекомбинантным аденовирусом человека 5-го серотипа.

Методы. Сборку целевого гена гемагглютинина *de novo* проводили методом полимеразной цепной реакции. Рекомбинантный аденовирус recAd5-swH1-His получали с помощью набора AdEasy™ Adenoviral Vector System, накопление препаративных количеств рекомбинантного белка проводили методом трансдукции recAd5-swH1-His суспензионной культуры клеток HEK293 в биореакторе волнового типа. Методом металл-хелатной аффинной очистки на сорбенте выделили рекомбинантный гемагглютинин из культуральной среды. Методами электрофореза и вестерн-блота определили его молекулярную массу, показали ее соответствие ожидаемой, а также наличие гистидиновых остатков. Методом непрямого иммуноферментного анализа со специфическими сыворотками установили антигенную специфичность swH1-His.

Результаты. В ходе работы получили рекомбинантный гемагглютинин swH1-His в количестве 1.2 мг из 50 мл культуральной жидкости, доказали соответствие его массы заявленной молекулярной массе (≈70 кДа) и наличие сшивки с гистидиновыми остатками, а также показали антигенную специфичность swH1-His в реакции с сыворотками.

Выводы. Определены физико-химическая и антигенная характеристики рекомбинантного белка гемагглютинина свиного гриппа A/H1N1 (swH1-His), полученного трансдукцией клеток HEK293 рекомбинантным аденовирусом человека 5-го серотипа. В дальнейшем полученный рекомбинантный гемагглютинин может быть использован как антиген для диагностики гриппа животных и человека.

Ключевые слова

рекомбинантный белок, рекомбинантный вирус человека 5-го серотипа, выделение рекомбинантного белка, антигенная специфичность, иммуноферментный анализ

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INTRODUCTION

Influenza is an acute respiratory infectious disease that circulates among the worldwide population, involving seasonal rises in morbidity. The high contagiousness and significant propensity to mutations of the influenza virus allow it to adapt quickly to the immunity formed by classical seasonal vaccines [1]. Therefore, the development of broad-spectrum influenza vaccines aimed at generating an immune response to conserved (low-covariable) sequences of influenza virus proteins remains relevant [2–5]. Such vaccines currently under active development in Russia [6, 7].

When assessing the effectiveness of vaccination, it is important to develop test systems for determining antibody titers in immunized people. Enzyme-linked immunosorbent assay (ELISA)-based test systems are well suited for this objective. Their integral parameter is the presence of an antigen that meets such criteria as availability of production and purification, as well as compliance of antigenic properties with the native protein.

Several different expression systems are currently available for the production of recombinant proteins. Although the choice of systems for recombinant protein production depends on the specific objectives, the most common goal is to obtain a sufficient amount of protein that is as similar in structure as possible to its natural form. Since protein glycosylation has a significant influence on physical (stacking, solubility, molecular weight, etc.) and biological (bioavailability, immunogenicity) properties of the obtained protein [8], the matching of the glycosylation profile of the synthesized recombinant protein to the native one often becomes a key factor.

The optimal expression system for obtaining complex eukaryotic recombinant proteins is mammalian cells. When expression conditions are close to natural, proteins can be obtained having a certain degree of glycosylation strongly approximating the native form [9]. This approach is particularly well suited for research tasks where convenient availability must be combined with the most natural form of recombinant proteins.

Among mammalian cells, there are two widely used cell lines as producers: the cell line derived from human embryonic kidney 293 (HEK293) cells and Chinese hamster ovary (CHO) cells. Although the latter option gives a high yield of recombinant protein (3–8 g/L) with complex glycosylation, it also associated with long and laborious work to breed a stable producer line. In an experimental laboratory, it is generally preferable to obtain protein by transient transfection, where CHO culture is inferior to HEK cells in terms of the complexity of transfection staging and the level of recombinant protein production [8]. HEK293 cells are also notable for the absence of oncogenic potential and low immunogenicity of the recombinant proteins produced due to the absence of structural modifications that are not characteristic of humans [10]. The variant of this line in the form of suspension has an undoubted advantage. When adapted to a serum-free medium, the suspension is optimal for scaling up the process and obtaining recombinant product without the excessive protein load (e.g., albumin) added by serum used in the cultivation of adherent HEK293 cells.

HEK293 is ubiquitously used to produce viral vectors. Due to the presence of adenoviral E1A/B genes responsible for auxiliary functions, adenoviral and adeno-associated viral vectors—in particular, human adenovirus serotype 5 (Ad5)—are produced in HEK293 cell culture [10].

While the production of recombinant proteins in vitro through transduction of cell culture with the human adenoviral vector Ad5 is not currently common, such an approach has considerable potential. Together with the advantages of the HEK293 suspension line, such a tandem can provide efficient production of recombinant protein requiring minimal economic costs and purification efforts.

In this study, we first produced a recombinant adenovirus (recAd5) expressing the hemagglutinin gene of swine influenza type A strain H1N1 (swH1-His) in the HEK293 cell line. After being affinity-purified on sorbent, the physicochemical and antigenic properties of the recombinant protein expressed in the culture medium were characterized.

MATERIALS AND METHODS

De novo assembly of the swine influenza hemagglutinin gene swH1-His

The *de novo* gene assembly was performed using the block method as described by Lei Young and Qihan Dong [11]. Oligonucleotides comprising sequential forward and reverse primers of 65 nucleotides each were selected for gene assembly (see Table 1). The primers were synthesized at *Eurogen* (Russia). The GeneRuller SM0311 DNA molecular weight marker (*Thermo Fisher Scientific*, USA) was used in this study.

At the first stage, four primers were taken into polymerase chain reaction (PCR) and combined into blocks of 200 base pairs (bp). For each reaction, the quantity of external primers contributed was 10 pmol and internal primers—0.01 pmol. Pfu DNA polymerase (*ELK Biotechnology*, China) was used. Primers were annealed at 55°C. After detecting amplicons by horizontal electrophoresis in 1.5% agarose gel (*Thermo Fisher Scientific*, USA), fragments were excised from the gel and purified on a single Cleanup mini column (*Evrogen*, Russia). Next, overlap extension polymerase chain reaction (OE-PCR) was performed using 100 ng of purified amplicons and Pfu polymerase. At the third stage, standard PCR was performed with primers ***swH1-His_out-F*** (5`-CGAGCCTAAGCTTCTAGATAAGATGCCGCCACCATGCTTGGACCCTGTATGCTGC-3`) and ***swH1-His_out-R*** (5`-TATCTAGATCCGGTGGATCGGATTCATTAGTGATGATGGTGATGGTGATGGTGATGG-3`).

The obtained swH1-His gene (full sequence is given in Table 2) was cloned by simple linear iterative clustering (SLIC) into the shuttle plasmid pShuttle-CMV hydrolyzed by Eco32I site from the AdEasy™ Adenoviral Vector System kit (*Stratagene*, USA) to obtain the plasmid pShuttle-swH1-His.

Production of recAd5 expressing the hemagglutinin gene of swine influenza type A strain H1N1 (swH1-His)

Lipofectamine® 2000 Reagent (*Thermo Fisher Scientific*, USA) was used for transfection according to the protocol. After transfection, cells were harvested, and three freeze-thaw cycles were performed to obtain viral efflux. The titer of the recombinant recAd5-swH1-His virus obtained was determined in the plaque formation reaction on HEK293 adherent cell culture at a confluence of 70%. The cytopathic effect (CPE) induced by recAd5-swH1-His was observed for 4 days.

To analyze the presence of sequences within the recAd5-swH1-His genome, DNA was isolated, and PCR carried out with product detection by agarose gel electrophoresis. The primer sequences for each sequence are presented in Table 3.

Accumulation of preparative amounts of swH1-His protein in culture fluid

To accumulate a preparative amount of recombinant hemagglutinin, the suspension cell line HEK293 was transduced with recAd5-swH1-His virus.

Adherent and suspension cell cultures of human embryonic kidney HEK293 were obtained from the Cell Culture Collection of the Tissue Culture Laboratory of the Tissue Culture Division of the D.I. Ivanovskiy Institute of Virology of the N.F. Gamaleya National Research Center of Epidemiology and Microbiology of the Ministry of Health of the Russian Federation. Cells were cultured in a wave-type bioreactor in Biostat® CultiBag RM 5L cell culture bags (*Sartorius AG*, Germany) in CDM4-HEK293 HyClone™ culture medium (*Cytiva*, USA) supplemented with 2 g/L sodium bicarbonate (*PanEco*, Russia), 1 g/L Poloxamer 188 (*Corning*, USA) and 4 mM L-glutamine (*PanEco*, Russia) at 37°C and 5% CO_2. After reaching a concentration of $1 \cdot 10^6$ cells/mL, the culture was aseptically supplemented with 10 mL of viral suspension containing recAd5-swH1-His adenovirus at a titer of 10^8 PFU/mL (plaque-forming units per mL) per 100 mL of medium.

Transduced cells were incubated for 3 days until 50–60% CPE was achieved. The percentage of live cells pre-stained with methylene blue was determined by visual counting in a Goryaev chamber (hemocytometer). After centrifuging the obtained cell suspension at 7700g for 20 min at room temperature (20°C), the supernatant was collected and stored at −70°C.

Metal-chelate affinity purification on sorbent

To a 50 mL volume of culture fluid containing the target protein, 1M potassium phosphate buffer (*Merck*, Germany) and sodium chloride (*Merck*, Germany) were added to a final concentration of 50 and 300 mM, respectively, and pH 8.0.

After washing 0.3 mL of the sorbent, Ni activated agarose (*Thermo Fisher Scientific*, USA) with 1 mL buffer A (50 mM potassium phosphate buffer with pH 8.0 and 300 mM sodium chloride), the sorbent precipitate was separated by centrifugation for 1 min at 3000 rpm. The procedure was repeated twice.

Following the transferal of the prepared sorbent was transferred to a tube with 50 mL of culture fluid containing 50 mM potassium phosphate buffer and 300 mM sodium chloride, the mixture was incubated on a shaker at 100 rpm for 16 h (overnight) at +22°C. Next, the supernatant was removed by centrifuging at 3000 rpm. The sorbent precipitate was then washed three times in 1 mL of buffer A.

Elution of protein from the sorbent was performed by rocking on a shaker at 180 rpm for 5 min using 1 mL of buffer B, consisting of 50 mM potassium phosphate buffer (pH 8.0) containing 300 mM sodium chloride and 250 mM methylimidazole (pH 12). After precipitating the sorbent by low-speed centrifugation (1 min at

3000 rpm), the supernatant was collected and dialyzed against 50 mM potassium phosphate buffer (pH 8.0).

The concentration of the affinity-purified protein antigen preparation was measured at 280 nm wavelength on a NanoDrop 2000 spectrophotometer (*Thermo Fisher Scientific*, USA). The preparation was stored at −20°C.

Electrophoretic analysis of swH1-His protein

Proteins were fractionated by electrophoresis in a 12% polyacrylate gel containing sodium dodecyl sulfate (SDS-PAGE) in Laemmli buffer system using a Mini-Protean 3 Cell vertical gel apparatus (*BIO-RAD*, USA). Protein molecular weight was determined using the PageRuler™ Plus Prestained Protein Ladder marker (*Thermo Fisher Scientific*, USA).

Samples for electrophoresis were applied at a loading rate of 1–4 μg of protein per track. Prior to being applied to the gel, samples were placed in denaturing (+) conditions using 2× Laemmli Sample Buffer (*Merck*, Germany), bringing the volume with distilled water to 40 μL (if necessary). Samples were heated at 95°C for 5–7 min and samples were added to the wells of the gel. A PageRuler™ Plus Prestained Protein Ladder molecular weight marker was used.

Electrophoresis was performed at a constant current of 20 mA/gel in SDS-PAGE Running Buffer, the components of which were 25 mM Tris-HCl, 0.25 M glycine and 0.1% sodium dodecyl sulfate (SDS), pH 8.3.

At the end of electrophoretic separation, the gel was scanned on a Gel Doc™ EZ device (*BIO-RAD*, USA), in the appropriate program to obtain a digital image and assess the electrophoretic purity of the preparation and the molecular weight of the target protein.

Western blot analysis of swH1-His protein

Following electrophoresis of the analyzed samples in SDS-PAGE, the proteins were transferred from the gel to the membrane by semi-dry transfer on a Trans-Blot® SD Semi-Dry Transfer Cell (*BIO-RAD*, USA) at a constant current of 250 mA over a 7 × 8 cm gel area.

At the end of transfer, 0.2 μm thick Nitrocellulose Membranes (*BIO-RAD*, USA) were washed three times in distilled water for 10 min each, then free sites on the membrane were blocked with working buffer: 20 mM phosphate buffered saline solution (PBS), pH 7.2–7.4, containing 0.1% polysorbate 20 (Tween 20) and 1% casein, for 30 min at 37°C on a shaker. The membrane was then incubated in the same buffer solution containing peroxidase-labeled mAb-a-His-HRP[1] monoclonal antibodies to histidine sequence (*Sigma-Aldrich*, Germany) at a working dilution of 1/5000 on a shaker at 22°C for 16 h (overnight). Following incubation, the membrane was first washed with distilled water, then washed three times for 10 min each with 20 mM PBS (pH 7.2) + 0.1% Tween 20 buffer on a shaker. Chemiluminescent detection of the target protein-conjugate complex was performed using Amersham ECL Prime Western Blotting Detection Reagents (*GE HealthCare*, USA) followed by immunoreplica scanning on an Amersham Imager 600 (*GE HealthCare*, USA).

Study of antigenic specificity of the target protein swH1-His in reaction with specific sera by indirect ELISA method

Indirect ELISA was performed according to the standard procedure. The obtained recombinant swH1-His protein and commercial recombinant hemagglutinin protein of influenza A virus strain H1N1/Salifornia/2009 (*Abcam*, UK) were used as antigen.

The sorption dose of the protein was 1 μg/mL in 50 mM K-carbonate buffer, pH 9.6. Blocking of unbound sites was performed with an inert protein solution—1% Blosker™ Casein (*Thermo Fisher Scientific*, USA). Antiviral monoclonal antibodies to human IgG—Goat anti-Human Fc-specific IgG HRP (*Sigma-Aldrich*, Germany)—were added at a working dilution of 1/5000. One-component TMB-substrate[2] (*Sigma-Aldrich*, Germany) was used for immune complex manifestation. Optical density was measured at wavelength λ = 450 nm using iEMS Reader MF Thermo Labsystems tablet spectrophotometer (*Thermo Fisher Scientific*, USA). The antibody titer was taken as the inverse of the highest serum dilution in which the optical absorbance was 2 times higher than background.

Statistical processing of the results

Statistical processing of the results was performed in GraphPad Prism program (*Dotmatics*, Canada). The geometric mean titer method was used for statistical processing with 95% confidence interval (CI).

1 HRP is horseradish peroxidase.

2 TMB is stabilized solution of 3,3',5,5'-tetramethylbenzidine hydrochloride.

RESULTS AND DISCUSSION

De novo assembly of the swine influenza hemagglutinin gene swH1-His

The hemagglutinin gene of Influenza A virus A/swine/Illinois/A01672343/2017(H1N1) strain (NCBI[3] Protein ID—AQU12415, NCBI GenBank—KY593239.1) was chosen to create the modified gene. The transmembrane domain and cytoplasmic domain were removed *in silico* and replaced with a spacer (GSSAS) and 10 codons encoding histidine. After optimizing the nucleotide sequence of the structural part of the swH1-His gene for the frequency of codon composition of *Homo sapience* genes, donor and acceptor splice sites, direct and inverted repeats, some restriction sites were removed.

Gene assembly was performed by PCR using the block method as described by Lei Young and Qihan Dong [11] with certain modifications: in the first step, a 200 bp amplicon was obtained from four primers (Fig. 1); at the second stage, the obtained short amplicons were assembled to a full-size gene, as a result of which all fragments complete each other, and at the third stage, a full-size DNA fragment of 1708 bp was amplified with external primers (Fig. 2) and cloned into a plasmid vector to obtain the pShuttle-swH1-His plasmid.

Production of recAd5 expressing the hemagglutinin gene of swine influenza type A strain H1N1 (swH1-His)

In the plaque formation reaction, the titer of recombinant adenovirus recAd5-swH1-His in the cell lysate was $2 \cdot 10^8$ PFU/mL.

PCR with product detection by agarose gel electrophoresis (Fig. 3) confirmed the presence of the swH1-His gene sequence in the DNA of recombinant human adenovirus serotype 5 (hexon), as well as the absence of the sequence of the E1 region of human adenovirus serotype 5.

Accumulation of preparative amounts of the target protein swH1-His in culture fluid

As shown in previous studies [12], the best parameters for accumulation of the target recombinant protein in the culture medium when using the adenovector system are an infection dose of 200 PFU/cell and 3 days of incubation with achievement of 50–60% CPE.

From the clarified culture fluid, 1 mL of a solution of recombinant swH1-His protein with a concentration of 1.2 mg/mL was obtained by metal-chelate affinity extraction in volume.

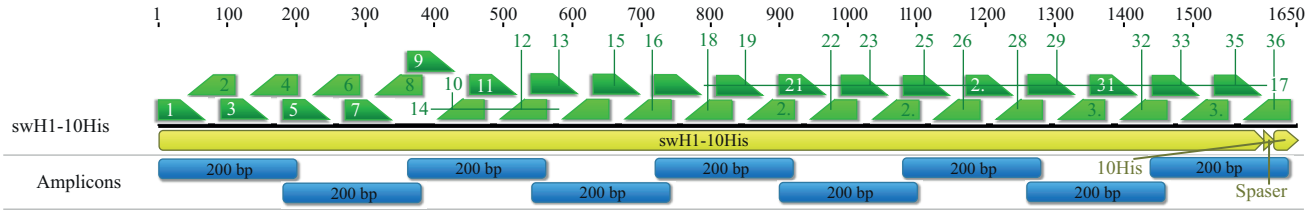

Fig. 1. Scheme for obtaining blocks of 200 base pairs (bp) using four primer amplification

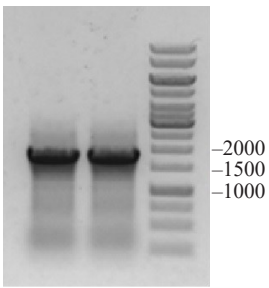

Fig. 2. Electropherogram of the results of amplification of the full-length swH1-His gene with terminal primers swH1-His_out-F and swH1-His_out-R. The amplicon size is 1708 bp, two repeats. M is the molecular weight marker

[3] NCBI — National Center for Biotechnology Information. https://www.ncbi.nlm.nih.gov/. Accessed June 10, 2023.

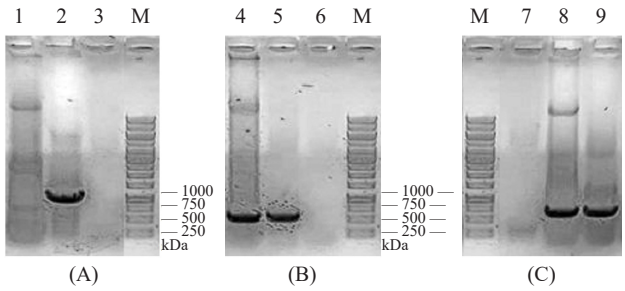

Fig. 3. Electropherogram of PCR analysis of DNA isolated from transduced cells with the recombinant adenovirus recAd5-swH1-His.
(A) PCR with primers for the E1 region of the adenovirus:
(1) recAd5-swH1-His,
(2) positive reaction control—1060 bp,
(3) negative reaction control.
(B) PCR with primers for adenovirus hexon:
(4) recAd5-swH1-His,
(5) positive reaction control—580 bp,
(6) negative reaction control.
(C) PCR with primers for the swH1-His gene:
(7) negative reaction control,
(8) recAd5-swH1-His,
(9) positive reaction control—670 bp.
M is the molecular weight marker

Physicochemical properties of recombinant swH1-His hemagglutinin

The obtained recombinant swH1-His protein was analyzed by protein electrophoresis in 12% SDS-PAGE under denaturing conditions. The molecular mass of the recombinant swH1-His protein and its conformity to the reported molecular mass were determined. The results of electrophoresis in SDS-PAGE are shown in Fig. 4.

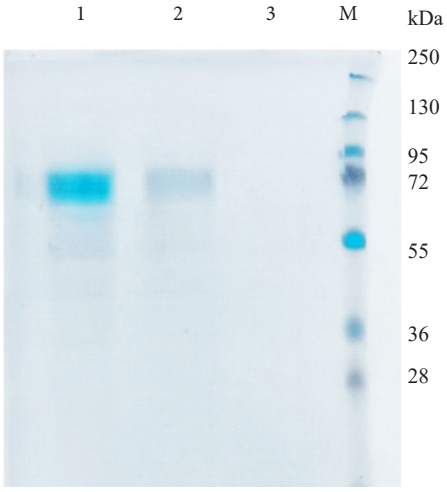

Fig. 4. Electropherogram of purified swH1-His protein:
(1) swH1-His protein (1st elution)—4 μg/track, (2) swH1-His protein (2nd elution)—1 μg/track, (3) "tailings" (sample after adsorption on Ni-agarose). M is the marker molecular weight

According to electrophoresis data, the molecular mass of the recombinant swH1-His protein is ≈70 kDa, which corresponds to the full-size hemagglutinin molecule of influenza A virus. The presence of 10 histidine residues in the purified recombinant swH1-His protein was confirmed by immunoblotting with monoclonal antibodies specific to the sequence of histidine residues (Fig. 5).

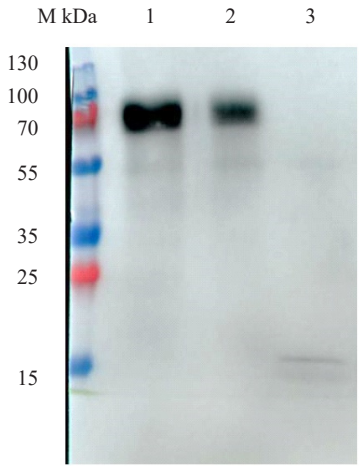

Fig. 5. Immunoreplica of purified swH1-His protein:
(1) swH1-His protein (1st elution)—4 μg/track, (2) swH1-His protein (2nd elution)—1 μg/track, (3) "tailings" (sample after adsorption on Ni-agarose). M is the marker molecular weight

Antigenic properties of recombinant swH1-His hemagglutinin

The antigenic activity of purified recombinant hemagglutinin swH1-His was characterized by indirect ELISA. For this purpose, specific sera of people who had had influenza were used. The diagnosis of influenza was made on the basis of clinical signs and positive PCR for influenza A/H1N1 RNA in a nasopharyngeal swab.

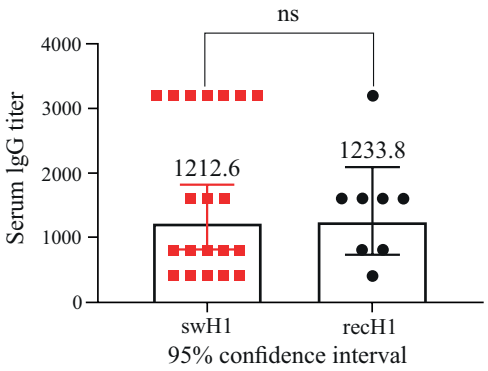

Fig. 6. Geometric mean titers of specific IgG in the blood sera of people who have had influenza to the swH1-His recombinant protein and to the recH1 commercial recombinant protein

During the period of recollection (on day 10–12 of the disease and later), serum was isolated from blood was obtained from patients using a standard method. All patients signed an informed consent form. The study complied with generally accepted medical and ethical standards, the principles of the Declaration of Helsinki of the World Medical Association (2013), and national laws and standards of the Russian Federation and the Rules of Good Clinical Practice (ICH GCP[4]), adopted in the Russian Federation (GOST R 52379-2005[5]).

In order to compare antigenic activity, serum samples were tested by indirect ELISA against the obtained swH1-His protein and commercial recombinant hemagglutinin protein of influenza A virus strain H1N1/California/2009 (recH1). The results of the indirect ELISA are shown in Fig. 6.

According to ELISA data, antigenic activity occurred in the swH1-His protein in reaction with sera of transfected people. However, the titers of specific antibodies to the obtained recombinant swH1-His antigen and to the branded commercial antigen recH1 did not show reliable differences. This indicates the potential possibility of using swH1-His as an antigen in an ELISA test system for the detection of specific antibodies.

CONCLUSIONS

In the course of this study, recombinant human adenovirus of the fifth serotype expressing the hemagglutinin gene of swine influenza type A strain H1N1 (swH1-His) containing 10 histidine residues was obtained by transduction of human embryonic kidney cell line HEK293.

The recombinant protein comprising hemagglutinin of swine influenza type A strain H1N1 (swH1-His) was affinity purified from the culture medium. The yield of highly purified swH1-His protein was 24 mg/L. The molecular mass of the purified protein was as reported (≈70 kDa); the authenticity of the cross-linking of the recombinant protein to the histidine sequence was confirmed by immunoblotting data.

Indirect ELISA confirmed the antigenic specificity of the swH1-His protein in reaction with specific blood sera of influenza-infected subjects. In future work, the obtained swH1-His protein will be used as an antigen in ELISA test system to assess the immunogenicity of vector vaccines for influenza prophylaxis.

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Authors’ contributions

E.D. Avdonina—protein purification by batch method, interpretation of the results of the study, writing and revising the text.

K.A. Pervoykina—gene synthesis and obtaining recombinant adenovirus.

L.V. Verkhovskaya—dot-blot, proteins electrophoresis in PAAG, Western blot.

D.N. Shcherbinin—gene design, synthesis, and production of recombinant adenovirus.

N.Yu. Viskova—obtaining preparative quantities of recombinant antibodies in culture fluid.

I.S. Kruzhkova—preparation of a bank of specific blood sera from patients who have had influenza A virus.

4 ICH GCP—International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use; ICH).

5 GOST R 52379-2005. National Standard of the Russian Federation. Good Clinical Practice. Moscow: Standartinform; 2006 (in Russ.).

M.A. Ilyina—preparation of a bank of specific blood sera from patients who have had influenza A virus.

L.V. Kudriavtseva—developing conditions for enzyme-linked immunosorbent assay with specific sera from patients who have had influenza A virus.

L.V. Kolobukhina—preparing the medical documentation for work with specific blood sera of patients who have had influenza A virus.

M.M. Shmarov—concept and design of the study, approval of the final version of the of the article for publication.

N.A. Antipyat—research idea, developing conditions for enzyme-linked immunosorbent assay with specific sera of blood of patients who have had influenza A virus.

A.L. Gintsburg—research idea and concept, approval of the final version of the article for publication.

I.N. Tyurin—concept and design of the study, coordination of medical documentation, approval of the final version of the article for publication.

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