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Stability of vaccine strains of seasonal live attenuated influenza vaccines when adapted to MDCK cell culture

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Abstract

Introduction. Currently, the vast majority of influenza vaccines in the world are produced using developing chicken embryos as substrate, but there is an urgent necessity for transferring vaccine production to continuous cell lines, which would ensure uninterrupted production during an avian influenza pandemic and also allow the vaccine to be administered to individuals with chicken protein allergies. When vaccine strains of live attenuated influenza vaccine (LAIV) grow in mammalian cells, adaptation mutations can occur that may affect the antigenic and immunogenic properties of the vaccine.

The aim of the study is to investigate the biological properties of vaccine strains of LAIV subtypes A/H1N1 and A/H3N2, produced by the classical reassortment in eggs, when adapted to Madin–Darby canine kidney (MDCK) cell culture.

Materials and methods. In current study, LAIV strains A/17/California/2009/38 (H1N1pdm09) and A/17/Texas/12/30 (H3N2) were used. These viruses were passaged on MDCK 5 times and the growth properties of the isolated clones by the plaque assay were analyzed *in vitro* and *in vivo*, also immunogenicity, cross-reactivity and protective efficacy were estimated on a mouse model, as well as using hyperimmune rat sera. Experimental series of LAIV strains A/17/Bolivia/2013/6585 (H1N1), A/17/Switzerland/2013/1 (H3N2) and B/60/Phuket/2013/26 were produced on MDCK cells at the Vector State Research Center of Virology and Biotechnology. The surface protein genes of monovalent vaccines were sequenced, and the mutations in HA and NA were identified and compared between adaptation to MDCK culture in laboratory and industrial conditions.

Results. Sequencing of surface antigens of MDCK-adapted variants of the A/H1N1 virus revealed adaptation mutations in the hemagglutinin molecule *N156D* (HA1 subunit) and *A44V* (HA2 subunit), which enhanced the replicative properties of the H1N1 vaccine strain in MDCK cells. The study of this MDCK-adapted variant in a mouse experiment showed no effect of the detected mutations on the immunogenic and protective properties of the vaccine. Adaptation of the H3N2 vaccine strain to MDCK cells resulted in a significantly higher number of substitutions in the HA molecule compared to the H1N1 virus, and it was shown that the *Y85E* and *N154K* mutations in HA2 are critical for virus multiplication in cell culture, and the set of mutations *P215T* in HA1 and *W92G, D160H* in HA2 gave the vaccine strain a significant advantage for growth in MDCK cells, which can be effectively used in the production of cell-based LAIVs.

Discussion. The study of the MDCK cell-produced series of LAIVs showed the presence of adaptation mutations in the hemagglutinin molecule of the H1N1 (*K116E* in the HA2 subunit) and H3N2 (*S219Y* and *N246K* in the HA1 subunit) strains. It is important to note that all the adaptation mutations studied did not affect the antigenicity of the vaccine strains.

Conclusion. In general, the data obtained in the course of the study indicate the feasibility of producing a culture-based live attenuated influenza vaccine from vaccine strains prepared by classical reassortment in eggs.

Keywords: *live attenuated influenza vaccine, MDCK cell line, adaptation mutations, hemagglutinin, antigenicity, immunogenicity*

ОРИГИНАЛЬНЫЕ ИССЛЕДОВАНИЯ

Стабильность вакцинных штаммов сезонной живой гриппозной вакцины при их адаптации к культуре клеток MDCK

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Аннотация

Введение. Подавляющее большинство гриппозных вакцин в мире производится с использованием развивающихся куриных эмбрионов (РКЭ) в качестве субстрата, однако активно обсуждается вопрос о переводе производства вакцин на перевиваемые клеточные линии, что обеспечит бесперебойность в условиях пандемии птичьего гриппа, а также позволит применять вакцину у лиц с аллергией на куриный белок. При накоплении вакцинных штаммов живой гриппозной вакцины (ЖГВ) в клетках млекопитающих могут возникать адаптационные мутации, влияющие на антигенные и иммуногенные свойства вакцины.

Цель работы — изучить биологические свойства вакцинных штаммов ЖГВ подтипов A/H1N1 и A/H3N2, полученных классическим способом в РКЭ, при их адаптации к культуре клеток почки собаки Мадина–Дарби (MDCK).

Материалы и методы. В работе были использованы штаммы для аттенуированной ЖГВ A/17/Калифорния/2009/38 (H1N1pdm09) и A/17/Техас/12/30 (H3N2). Мы провели серийное пассирование этих вирусов на MDCK и проанализировали ростовые свойства изолированных методом бляшек клонов *in vitro* и *in vivo*, их иммуногенность, перекрёстную реактивность и защитную эффективность на модели мышей, а также с использованием гипериммунных крысиных сывороток. Экспериментальные серии вакцинных штаммов ЖГВ A/17/Боливия/2013/6585 (H1N1), A/17/Швейцария/2013/1 (H3N2) и B/60/Пхукет/2013/26 были наработаны на культуре MDCK в ГНЦ ВБ «Вектор». Мы провели секвенирование генов поверхностных белков клеточных моновакцин и сравнили мутации, обнаруженные в гемагглютинине и нейраминидазе при адаптации к культуре клеток MDCK в лабораторных и производственных условиях.

Результаты. Секвенирование поверхностных антигенов MDCK-адаптированных вариантов вируса A/H1N1 обнаружило адаптационные мутации в молекуле гемагглютинина — *N156D* (субъединица HA1) и *A44V* (субъединица HA2), одновременное присутствие которых усиливало репликативные свойства вакцинного штамма ЖГВ H1N1 в культуре клеток MDCK. Изучение данного адаптированного к культуре клеток MDCK варианта в эксперименте на мышах не выявило влияния обнаруженных мутаций на иммуногенные и протективные свойства вакцины. Адаптация вакцинного штамма ЖГВ H3N2 к культуре клеток MDCK привела к появлению существенно большего количества замен в молекуле HA, по сравнению с вирусом H1N1. Мутации *Y85E* и *N154K* в HA2 являются критическими для размножения вируса в культуре клеток, а набор мутаций *P215T* в HA1 и *W92G*, *D160H* в HA2 дали вакцинному штамму существенное преимущество для размножения в культуре клеток MDCK, что может быть эффективно использовано в производстве культуральной ЖГВ.

Обсуждение. Изучение производственных серий культуральных ЖГВ показало наличие адаптационных мутаций в молекуле гемагглютинина штаммов H1N1 (*K116E* в субъединице HA2) и H3N2 (*S219Y* и *N246K* в субъединице HA1). Все изученные адаптационные мутации не влияли на антигенность вакцинных штаммов.

Заключение. Полученные в ходе исследования данные указывают на перспективность производства культуральной ЖГВ из реассортантных штаммов, подготовленных стандартным путём в РКЭ.

Ключевые слова: *живая гриппозная вакцина, культура клеток MDCK, адаптационные мутации, гемагглютинин, антигенность, иммуногенность*

Introduction

Annual vaccination against seasonal influenza is the most effective way to combat this infection [1]. For over 70 years, the allantoic fluid of the embryonated chicken eggs has been recognized as the most optimal substrate for receiving of high-yield virus material, which made it possible to produce a sufficient number of vaccine doses for seasonal flu vaccine campaign [2, 3]. However, the outbreak of highly pathogenic H5N1 avian influenza in Hong Kong in 1997, and since 2003, the widespread spread of this infection throughout the world, forced the scientific community to consider switching the production of influenza vaccines to immortalized cell lines, since such production would not depend on the promptly supply of embryonated chicken eggs in the avian influenza presence [4]. Furthermore, the production of vaccine viruses in cell culture allows avoiding the occurrence of egg-adaptation mutations in vaccine strains, which could have a negative impact on the antigenicity and immunogenicity of the vaccine and, as a result, reduce its protection effectiveness [5, 6]. Generally accepted cell line for the influenza vaccines production is the Madin-Darby canine kidney (MDCK) cell culture, which is currently used to produce the Flu-CellVax — cultural inactivated influenza vaccine [7]. Another important advantage of the culture influenza vaccine is the possibility of its use in people suffering from an egg allergy.

The preparation of vaccine strains for a Russian licensed live attenuated influenza vaccine (LAIV) is currently possible only by classical reassortment in embryonated chicken eggs, in which the 6:2 vaccine formula is obtained according to a proven protocol and usually allows obtaining the required vaccine strain [8]. To transfer LAIV production from chicken eggs to MDCK cell culture, studies were previously conducted to obtain vaccine strains in MDCK cells. It was shown that during classical reassortment in this cell line of the epidemic virus and the attenuation donor, there was no formation of a vaccine strain with the required 6:2 genome formula [9]. Since 2019, the World Health Organization has divided strains for cell-based and egg-based influenza vaccines in its recommendations for current influenza virus strains for seasonal influenza vaccines because of the fact that many studies have shown that strains isolated on the same substrate should be used for vaccine production in order to avoid the appearance of adaptive mutations [10]. The issue of obtaining LAIV strains in MDCK cells could be resolved using reverse genetics methods to assemble influenza viruses with a given set of genes, however, the presence of a patent for this technology by MedImmune excludes the possibility of its use for commercial production [11]. Thus, one of the options for obtaining culture LAIV is the use of vaccine strains obtained by classical reassortment in developing chicken embryos for their growth on a cell line. Since the substrate for the accumulation of vaccine viruses is exchanged, it is necessary to study the stability of the main biological properties of LAIV strains during their adaptation to MDCK cells.

The aim of this study was to identify possible adaptation mutations in the LAIV strains A/H1N1 and A/H3N2 obtained by the classical reassortment method in the emryonated chicken eggs during their adaptation to the MDCK cells, with subsequent assessment of the effect of the detected mutations on the growth characteristics of the viruses, their immunogenicity, cross-reactivity and protective activity in the experiment. Furthermore, as part of the study, an experimental series of cultural LAIV produced by the State Research Center of Virology and Biotechnology Vector on MDCK cell culture using industrial bioreactors was analyzed [12].

Materials and methods

Viruses

The LAIV strains A/17/California/2009/38 (H1N1pdm09) (Calif17) [13] and A/17/Texas/12/30 (H3N2) (Tex17) [14] were used, the surface genes of which corresponded to the epidemic strains A/California/07/2009 (H1N1pdm09) (NCBI database number: NC_026433 (HA) and NC_026434 (NA)) and A/Texas/50/2012 (H3N2) (NCBI database number: KC892248 (HA) and KC892237 (NA)) respectively. Mouse-adapted influenza virus A/California/07/09 (H1N1) was obtained from the Collection of Influenza and ARVI Viruses of the A.A. Smorodintsev Research Institute of Influenza and were used for the challenge experiment [15]. Experimental series of LAIV strains A/17/Bolivia/2013/6585 (H1N1), A/17/Switzerland/2013/1 (H3N2) and B/60/Phuket/2013/26 were produced on the MDCK cells at the Vector State Research Center of Virology and Biotechnology using bioreactors with a culture vessels volume of 2 liters (Multigen) and 10 liters (Biostat) [12].

Virus growth in eggs and MDCK cells and infectious titer counting

To grow influenza viruses in eggs, 10–11-day-old embryonated chicken eggs were infected with 0.2 ml of viral liquid, after which the eggs were incubated for 48 h at 33°C. Virus propagation in MDCK cells was carried out on a 24-hour monolayer with 90–95% cells confluence, grown in DMEM with 1× antibiotic-antimycotic (AA) (Gibco) and 10% fetal bovine serum (FBS) (Biolot) at 37°C in a incubator with 5% CO_2. To infect MDCK cell culture, the monolayer was washed twice with a warm solution of phosphate-buffered saline (PBS), after which the viral suspension was added in a volume of 1, 2, 4 ml to T-25, T-75 and T-175 flasks, respectively. After contact for 1 h at 33°C and 5% CO_2, the inoculum was removed and condition medium (DMEM with 1×AA and 1 µg/ml TPCK trypsin (Sigma-Aldrich)) was added. After 72 h of incubation at 33°C, the cytopathic effect of the virus was visually

assessed and its titer was counted in the hemagglutination assay using the standard method with chicken RBC. Infectious titers of viruses in both cultivation systems were determined by the limiting dilution assay. The titer in MDCK cells was evaluated at 96-well plates with a daily monolayer, while serial 10-fold dilutions were prepared in condition medium. After 1 h adsorption at 33°C, the inoculum was removed, the cells were washed and then incubated in a maintenance medium for 3 days at 33°C and 5% CO_2. The presence of viruses in the wells was determined in a hemagglutination assay with chicken RBC. Viral titers in eggs and MDCK cells were counted using the Reed and Mench method [16] and put as 50% embryonated or tissue culture infectious doses ($lgEID_{50}$/ml and $lgTCID_{50}$/ml).

Virus adaptation to MDCK cells

Adaptation of influenza viruses to MDCK cell culture was performed over 5 serial viral passages at an optimal multiplicity of infection (MOI) of 0.001–0.010, followed by virus cloning using the plaque assay. For this purpose, 10-fold dilutions of viruses were applied in double replicates to 6-well plates seeded with MDCK cells the day before. After an hour of contact with regular plates shaking, the inoculum was removed and 3 ml of an agar coating obtained by mixing equal volumes of 2×DMEM medium (in the presence of 2×AA and 2 μg/ml TPCK trypsin) and 1.6% low-melting agarose (Lonza) were added to the wells. On the 3rd–5th day of incubation, the viral plaques were visually observed, 20–30 plaques that were easily separated from each other were isolated at limiting dilutions, separate clones of the virus was isolated from each plaque, which was then grew on the MDCK cell culture. Each propagated viral clone was whole-genome sequenced by the Sanger method using the BigDye Terminator Cycle Sequencing Kit v3.1 (Thermo). First of all, the presence of unique attenuating mutations of LAIV strains was checked, since it is their existence that determines the attenuating of LAIV and the possibilities of its use [17], and then a search was conducted for amino acid substitutions in the genes of surface proteins: hemagglutinin (HA) and neuraminidase (NA).

Animal experiments

In animal experiments, CBA mice (Stolbovaya, Russia) were used. The study was approved by the Ethics Committee of the Institute of Experimental Medicine (protocol No. 1/20 dated 02/27/2020). To assess the immunogenicity and protective efficacy of the H1N1 vaccine strains, female CBA mice were immunized intranasally at a dose of 10^6 $TCID_{50}$ in a 50 μl volume , twice with a difference of 3 weeks, after which, after another 21 days, blood serum was collected to assess antibody levels in the hemagglutination inhibition assay (HAI) and enzyme-linked immunosorbent assay (ELISA) using a standard methods. To study the pro-

tective efficacy, an experiment was carried out on mice immunized according to the scheme described above, followed by infection with a lethal dose 5.0 lg EID_{50} of the mouse-adapted A/California/07/09 (H1N1) strain with daily monitoring of survival for 14 days. To assess the cross-reactivity of antibodies produced to the H3N2 LAIV strains, hyperimmune rat sera were obtained. For this purpose, Wistar rats (Rappolovo, Russia) were immunized 5 times with an interval of 5–7 days. The first, third and fifth immunizations were administered intraperitoneally in a volume of 5 ml/animal, the second and fourth immunizations were administered subcutaneously in the withers using complete Freund's adjuvant in a volume of 1 ml/rat, the ratio of virus and adjuvant was 1 : 1. A week after the last immunization, whole blood samples were taken from all immunized animals. After centrifugation of the blood for 15 min at 3000g, the serum was carefully collected, and aliquots were stored at –20°C.

Immunological methods

The animal blood serum was studied in HAI according to the standard WHO protocol with chicken RBC and treatment of the serum with a receptor-destroying enzyme (Denka). The serum titer in HAI was counted as the last dilution at which complete inhibition of erythrocyte agglutination was observed.

ELISA with mouse serum samples was performed using the relevant wild influenza viruses purified on a sucrose density gradient as the antigen. The 16 agglutinating units (AU) of antigen were added to 96-well plates with high sorption (Corning) 50 μl/well and incubated overnight at 4°C. The plates were washed 3 times with washing buffer (PBS + 0.05% Tween-20 (Biolot)), unbound sites were blocked with 1% bovine serum albumin. Two-fold dilutions of sera were prepared in separate U-bottom plates, which were then transferred to the wells of the ELISA plate washed from the blocking solution. After incubation for 1 h at 37°C, the plates were washed 3 times with washing buffer, dried, and secondary antibodies conjugated with horseradish peroxidase, anti-mouse IgG were added in dilution ration 1 : 10,000. The plates were incubated for 1 h at 37°C, washed 5 times with washing buffer, dried, and 50 μl/well of TMB substrate (Thermo) were added, which was incubated in the dark for up to 20 min at room temperature. The reaction was stopped by adding 50 μl of 1 M H_2SO_4. The primary ELISA results were recorded on a spectrophotometer (Bio-Rad) at a wavelength of 450 nm. The area under the curve parameter of optical density was calculated using the GraphPad Prizm v. 7 software package.

Statistical data analysis

Statistical processing was performed using the GraphPad Prizm v. 7 software. For pairwise comparison of virus titers, the nonparametric Mann-Whitney

U-test was used; antibody levels between groups of immunized mice were compared in a nonparametric one-way ANOVA with the Kruskal-Wallis correction. Differences were considered significant at $p < 0.05$.

Results

Adaptation of the H1N1 vaccine strain to MDCK cells in laboratory conditions

As a result of serial passaging of the Calif17 vaccine strain in MDCK cell culture with subsequent cloning by the plaque method, 21 virus variants were obtained. Sequencing showed the safeguarding of unique attenuating mutations in the genes of internal and non-structural proteins. At the same time, only 2 of the 21 studied variants did not have amino acid substitutions in the HA molecule, and in the remaining isolates, either *N156D* mutations in HA1, or *A44V* in HA2, or both were detected simultaneously (**Table 1**).

The arrangement of amino acid substitutions in the HA molecule of Calif-17 cell clones is shown in **Fig. 1**.

The growth capacity of three MDCK-adapted variants (Calif17-1, Calif17-4, and Calif17-8) with different sets of found mutations were studied in eggs and MDCK cells in comparison with the original virus propagated in eggs (Calif17). Reproduction of the original Calif17 vaccine strain on different substrates also differed by almost two orders of magnitude: the virus titer in eggs was 8.0 lg EID_{50}/ml, while the virus titer in MDCK cells was 6.3 lg $TCID_{50}$/ml (**Fig. 2**). In the study of MDCK-adapted variants of the Calif17 strain, a reliable increase in virus titers in MDCK cell culture by more than 10 times was found in 2 strains (Calif17-4 — 7.3 lg $TCID_{50}$/ml and Calif17-8 — 7.6 lg $TCID_{50}$/ml), both containing the *A44V* mutation in HA2. The Calif17-8 variant also retained a high level of reproduction in embryonated chicken eggs (8.2 lg EID_{50}/ml), while the Calif17-1 strain, which has only the N156D mutation in HA1, did not improve reproduction in cell culture and decreased it in eggs by more than 30 times (6.2 lg $TCID_{50}$/ml and 6.6 log EID_{50}/ml). Thus, the *A44V* mutation in HA2 has a positive effect on the replication of the H1N1pdm09 vaccine strain in both culture systems.

The isolated variants of the Calif17 virus after plaque cloning, carrying one of the two mutations, acquired both mutations during subsequent accumulation in the MDCK cell — *A44V* in HA2 and *N156D* in HA1,

becoming identical in amino acid composition to the Calif17-8 variant. This phenomenon suggests that these two mutations are strongly associated with the adaptation of the virus to the mammalian cell line, but the initial five-times passaging did not completely replace the corresponding amino acids in the heterogeneous virus population. Additional growing of variants with one mutation in the MDCK cells continued the process of virus adaptation, introducing the missing mutation in the HA gene. Thus, in the experiment on the immunogenicity and protective activity of viruses in mice, it was possible to compare only the Calif17-8 strain, carrying both adaptation mutations, with the original Calif17 virus propagated in eggs.

Figure 3 shows the results of ELISA of blood serum from immunized mice, where the epidemic strain A/California/09/07 (H1N1pdm09), grew in the eggs and purified on a sucrose density gradient, served as an antigen substrate. Statistical processing of the experimental data showed that there are no significant differences between levels of IgG antibodies induced by both vaccine strains, which indicates the absence of a negative effect of the detected adaptation mutations *A44V* in HA2 and *N156D* in HA1 on the immunogenicity of LAIV H1N1pdm09.
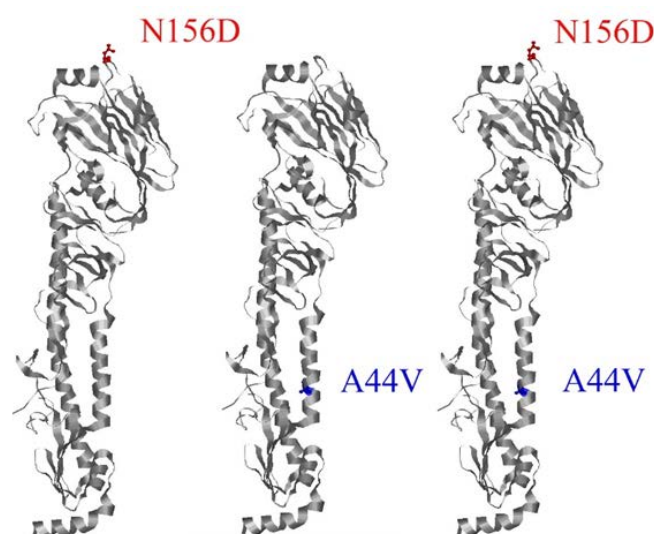
Fig. 1. Mapping of adaptation amino acid substitutions in the HA molecule of the vaccine strain Calif17.

The arrangement of amino acids is shown on the HA monomer A/California/04/2009 (H1N1) (PDBID: 3UYX). Illustrations were obtained using the «RasMol v. 2.7.5» program.

Table 1. Mutations found in the HA molecule of MDCK-adapted variants of the Calif17 vaccine strain

HA subunit	Amino acid position	Original virus Calif17	MDCK-adapted viral variants		
			Calif17-1 (9 clones)	Calif17-4 (7 clones)	Calif17-8 (3 clones)
HA1	156	Asn	Asp	Asn	Asp
HA2	44	Ala	Ala	Val	Val

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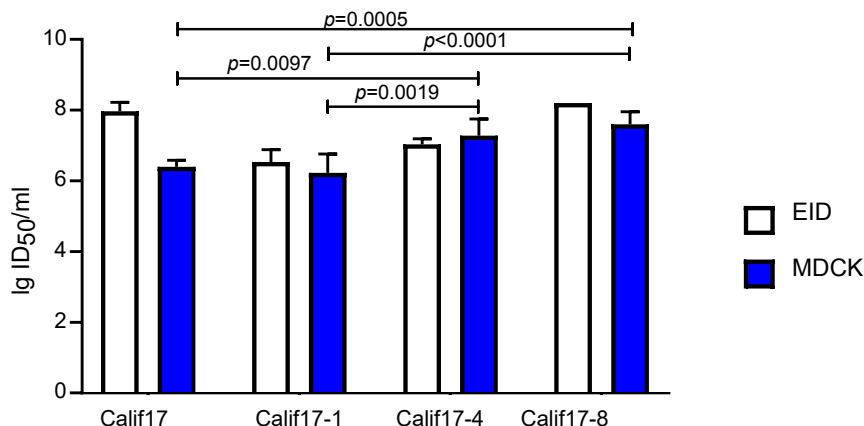**Fig. 2.** Infectious titers of the LAIV strain A/17//California/2009/38 (H1N1pdm09) and its MDCK-adapted variants
in embryonated chicken eggs and MDCK cells.
*$p < 0.05$ compared with $TCID_{50}$/ml Calif17; †$p < 0.05$ compared with EID_{50}/ml Calif17.

The cross-reactivity of antibodies induced by the MDCK-adapted variant of Calif17-8 and the vaccine strain Calif17 was assessed in HAI using the classical method. Adaptation mutations did not affect the ability of the produced antibodies to bind the original HA variant of the A/California/07/09 (H1N1pdm09) strain (**Table 2**), which is completely consistent with the immunogenicity data presented above.

The effect of the adaptation mutations *N156D* in HA1 and *A44V* in HA2 on the protective efficacy was studied using the wild virus A/California/07/09 (H1N1pdm) adapted to mice. In the challenge experiment, mice immunized with the vaccine strains Calif17 and Calif17-8, as well as the control group of naive animals, were infected with the mouse-adapted lethal virus at a dose of 10^5 $lgEID_{50}$, after which daily survival monitoring have been carrying out for 14 days. The protective efficacy of both the egg-derived LAIV strain and its MDCK-adapted variant was 100%, while the animals of the control group died completely, and the survival rate in it was 0% (Fig. 3, *c*, *d*). Thus, the studied MDCK adapted mutations did not affect the protective efficacy of the LAIV strain Calif17.
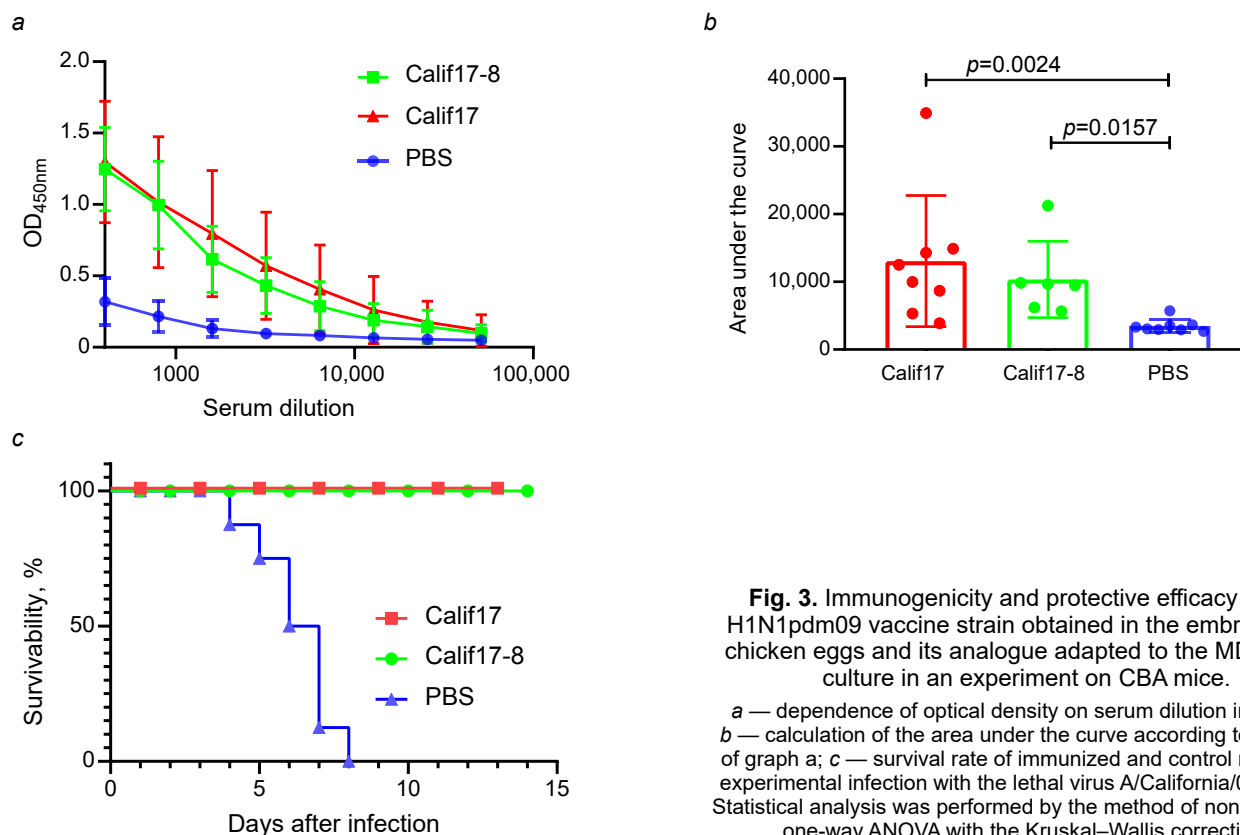
Fig. 3. Immunogenicity and protective efficacy of the H1N1pdm09 vaccine strain obtained in the embryonated chicken eggs and its analogue adapted to the MDCK cell culture in an experiment on CBA mice.

a — dependence of optical density on serum dilution in ELISA; *b* — calculation of the area under the curve according to the data of graph a; *c* — survival rate of immunized and control mice after experimental infection with the lethal virus A/California/09/07 MA. Statistical analysis was performed by the method of nonparametric one-way ANOVA with the Kruskal–Wallis correction.

Table 2. Cross-reactivity of antibodies produced in response to immunization of mice with different variants of LAIV H1N1pdm09

Antigens	HAI titer of antibodies in blood sera of mice vaccinated with the H1N1 viral variants	
	Calif17	Calif17-8
Calif-wt	640	320
Calif17	640	320
Calif17-8	320	320

Adaptation of the H3N2 vaccine strain to MDCK cells in laboratory conditions

The vaccine strain A/17/Texas/12/30 (H3N2) (Tex17) containing HA and NA from the epidemic influenza virus A/Texas/50/2012 (H3N2) was used in the study. After adaptation of Tex17 to the MDCK cell culture, 20 clones of Tex17 were isolated, 10 of which were found to have various amino acid differences in the HA molecule during sequencing (**Table 3**).

No matching mutations were found in the HA molecule of MDCK-adapted clones of the vaccine strain Tex17, but frequently occurring amino acid substitutions were noted: V176I, P215T, P221S and D265E in HA1. Single mutations were also identified: *R301K* in HA1, *D79N, D79G, Y83H, E85D, W92G, K124E, N154K, D160H* and *N169K* in HA2 (Table 3). The localization of frequently occurring and single mutations is different: the former are located in the globular part of HA near the receptor-binding site, the latter are in the HA stalk-domain.

An examination of the vaccine strain reproduction showed that the original vaccine strain Tex17 reproduces 50 times better in the eggs than in the MDCK cells. Interestingly, one of the MDCK-adapted variants, strain Tex17-16, showed the most significant increase in infectious titer in MDCK cells compared to the egg-derived vaccine strain Tex17 (**Fig. 4**). Thus, the set of mutations *V176I, P221S, D265E* in HA1 and *N154K* in HA2 is associated with an increase in the reproductive capacity of the vaccine strain in MDCK cell culture.

Furthermore, the variant Tex17-8 was identified, which had a significantly lower titer in the MDCK cell culture compared to the control strain Tex17, which was affected by the amino acid substitutions *P221S, D265E* in HA1 and *Y85E* in HA2. Thus, it follows that the *N154K* and *Y85E* mutations in HA2 have critical for virus replication in cell culture and require further study. The strain Tex17-17, which replicated 200 times better in the MDCK cell culture than in the developing chicken embryo system, also turned out to be interesting. This means that the adaptation mutations *P215T* in HA1 and *W92G, D160H* in HA2 gave the vaccine strain a significant advantage for replication in the MDCK cell culture, which can be effectively used in the production of cell-based LAIV. However, the set of the specified mutations significantly reduced the reproduction of viruses in the eggs, and if we pay attention to the viruses Tex17-11 and Tex17-15, we can see that these variants differ only in mutations in the HA2 subunit of the HA molecule, and it is the amino acid differences in the HA stalk-domain that are key in the adaptation of H3N2 vaccine strains to the MDCK cells.

Table 3. Mutations found in the HA molecule of MDCK-adapted variants of the Tex17 vaccine strain

HA Subunits	Amino acid position	Original virus	MDCK-adapted variants									
			Tex17-1	Tex17-4	Tex17-8	Tex17-9	Tex17-11	Tex17-15	Tex17-16	Tex17-17	Tex17-20	Tex17-21
HA1	176	Val	Val	Val	**Ile**	**Ile**	Val	Val	**Ile**	Val	Val	**Ile**
	215	Pro	Pro	Pro	Pro	Pro	**Thr**	**Thr**	Pro	**Thr**	Pro	Pro
	221	Pro	Pro	Pro	**Ser**	**Ser**	Pro	Pro	**Ser**	Pro	Pro	**Ser**
	265	Asp	Asp	Asp	**Asp/Glu**	**Asp/Glu**	**Asp/Glu**	**Asp/Glu**	**Asp/Glu**	Asp	Asp	Asp
	301	Arg	Arg	Arg	Arg	Arg	Arg	Arg	Arg	Arg	**Lys**	Arg
HA2	79	Asp	Asp	Asp	Asp	Gly	Asp	Asp	Asp	Asp	Asp	**Asn**
	83	Tyr	**His**	Tyr	Tyr	Tyr	Tyr	Tyr	Tyr	Tyr	Tyr	Tyr
	85	Glu	Glu	Glu	**Asp**	Glu	Glu	Glu	Glu	Glu	Glu	Glu
	92	Trp	Trp	Trp	Trp	Trp	Trp	Trp	Trp	**Trp/Gly**	Trp	Trp
	124	Lys	Lys	**Glu**	Lys	Lys	Lys	Lys	Lys	Lys	Lys	Lys
	154	Asn	Asn	Asn	Asn	Asn	Asn	Asn	**Lys**	Asn	Asn	Asn
	160	Asp	Asp	Asp	Asp	Asp	Asp	Asp	Asp	**His**	Asp	Asp
	169	Asn	Asn	Asn	Asn	Asn	Asn	**Lys**	Asn	Asn	Asn	Asn

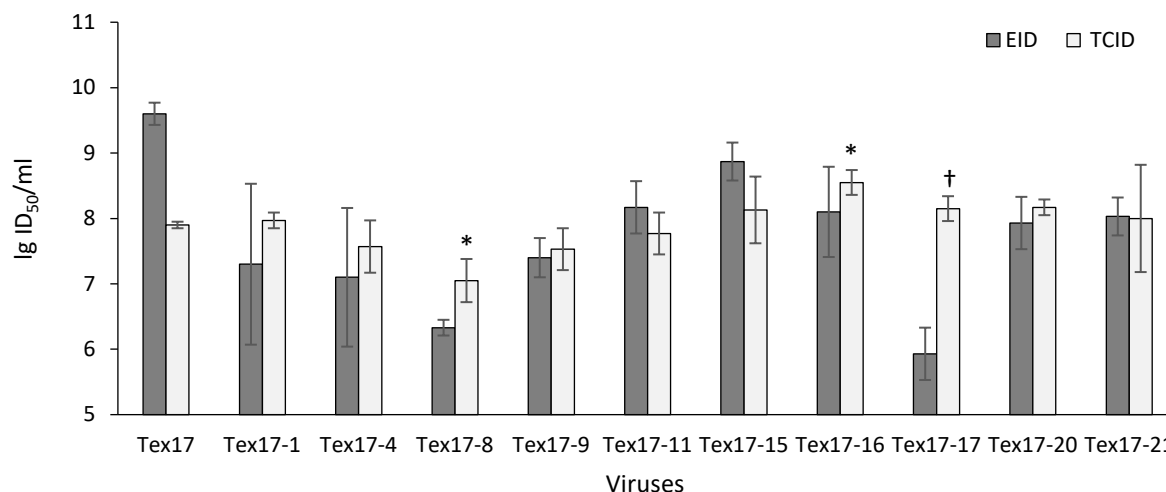**Note:** MDCK-adapted amino acid substitutions in HA have been highlighted compared to the egg-derived Tex17 vaccine strain.

Fig. 4. Reproduction of influenza A/17/Texas/12/30 (H3N2) vaccine viruses in the different culture systems.
*$p < 0.05$ compared with $TCID_{50}$/ml Tex17; †$p < 0.05$ compared with EID_{50}/ml Tex17-17.

Figure 5 shows the three-dimensional structures of the HA molecules of the vaccine strains Tex17-8 and Tex17-16, on which the noticed adaptation mutations are marked. It is evident that the mutations that critically affected the level of reproduction of vaccine viruses in the MDCK cells are located in the HA stalk-domain. It is also of interest to study the immunogenicity and antigenicity of these viruses, but current influenza viruses of the H3N2 subtype are not able to infect mice, therefore, within the framework, study of the immunogenicity of H3N2 viruses was not carried out in a mouse model. To assess the antigenicity of the most interesting MDCK-adapted variants, hyperimmune rat sera were obtained.

The cross-reactivity of antibodies induced by the cell clones Tex17-8, Tex17-16 and Tex17-17 was assessed in HAI using the classical method. It was shown that adaptation mutations did not affect the ability of the produced antibodies to bind the HA, of the egg-derived A/Texas/50/2012 (H3N2) strain (**Table 4**).

Adaptation of trivalent LAIV vaccine strains to MDCK cell culture under industrial production conditions

In Russia, cultured LAIV is being developed at the State Research Center of Virology and Biotechnology Vector, using reassortant LAIV vaccine strains prepared by the classical reassortment method at the developing chicken embryos as the starting material. The company registered a system for producing LAIV in mammalian cell culture and successfully produced an experimental series of cellular monovalent vaccines against seasonal influenza viruses in 2015 [12] and then conducted phase I clinical trials [18]. However, it remained unknown how the production process affected the properties of the LAIV strains. Sequencing of the HA and NA surface protein genes of cellular monovalent vaccines showed that adaptation mutations appeared only in the HA molecules of influenza A viruses, while no amino acid substitutions were found in the B/60/Phuket/2013/26 strain (**Table 5**). Thus, in the vaccine strain A/17/Bolivia/2013/6585 (H1N1pdm09), the *K116E* mutation in the HA2 subunit was identified, and in the strain A/17/Switzerland/2013/1 (H3N2), the *S219Y* and *N246K* mutations in the HA1 subunit were identified.

In **Fig. 6**, the positions at which amino acid substitutions were detected during adaptation of vaccine strains to cell culture on a laboratory scale (*A44V* in HA2 of the H1N1 subtype, *V176I, P215T, P221S* and *D265E* in HA1 of the H3N2 subtype) and during the production of culture LAIV batches (*K116E* in HA2 of the H1N1 subtype, *S219Y* and *N246K* in HA1 of the H3N2 subtype) are highlighted on the monomers of the corresponding HA molecules. It should be noted that, although the mutations detected are not identical in different viruses, their common localization can be observed in each subtype. Thus, positions 44 and 116 in HA2 of the H1N1 subtype are in close proximity to each other, positions 176, 215, 219, 221 in HA1 of the H3N2 subtype are localized in the region of the receptor-binding site.

Discussion

Adaptation of the virus to a new host range inevitably entails the emergence of adaptive mutations, which can lead to changes in the properties of the original virus. In this case, adaptive mutations can be dramatic, as, for example, occurred during the adaptation of influenza viruses from avian cells to mammalian cells, which led to the H2N2 influenza pandemics in 1957 and H3N2 in 1968 [19–22]. The substrate for the production of biomass for obtaining an influenza vaccine, both live and inactivated, can also affect its properties. As mentioned above, the preparation of strains for Russian LAIV is possible only in the embryonated chicken eggs, while

there are known cases of the emergence of egg-adaptive mutations that affected the antigenicity of the vaccine strain, which led to a decrease in the effectiveness of the vaccine during its mass use [21, 23–25]. Since A/H3N2 strains are the most variable and more susceptible to antigenic drift than others, this circumstance must be taken into account when producing vaccine viruses in various culture substrates [21]. Thus, from 2013 to 2018, a number of mutations were discovered that critically affected the antigenicity of circulating viruses: the *F159Y* and *K160T* substitutions in antigenic site B, together with the N residue in site 158, contribute to the acquisition of an additional glycosylation site, which actively helps the virus evade the immune response [26–28]. Then, in the process of evolution, a characteristic amino acid substitution *N171K* occurred, located on the antigenic site D of the HA molecule, as well as the *D122N* and *T135K* substitutions in antigenic site A, each of which causes the loss of N-linked glycosylation sites [29]. The following season revealed the presence of a *S144K* substitution in the HA1 molecule, which is located in the antigenic site flanking the receptor-binding site. Two clusters of the H3N2 influenza virus showed that the *I58V* and *S219Y* substitutions, as well as the *122D* and *S262N* substitutions, which lead to the loss of the glycosylation site, are also antigenically important [21]. In the current study, during adaptation to MDCK cells, mutations were found that are also probably located in antigenic sites: *V176I, P215T, P221S* and *S219Y*, however, it was shown that they
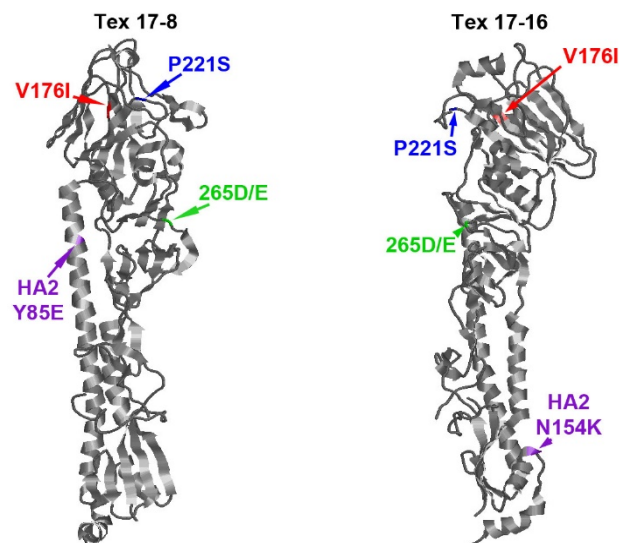

Fig. 5. Mapping of adaptation amino acid substitutions in the HA molecule of the vaccine strain Tex17.

The arrangement of amino acids is shown on the HA monomer of A/Pennsylvania/14/2010 (H3N2) (PDBID: 6MZK). Illustrations were obtained using the «RasMol v. 2.7.5» program

do not affect the antigenicity of the vaccine strain, but affect its ability to grow in mammalian cells.

Studies of the infectious activity and stability of high-yielding influenza virus strains for inactivated influenza vaccine demonstrated that cultivation of H1N1 and H3N2 influenza viruses in Vero or MDCK cell culture under acidified conditions maintains virus stability, which was successfully used in the production of inac-

Table 4. Cross-reactivity of antibodies in hyperimmune rat sera obtained to different variants of the LAIV H3N2 vaccine strain

Antigens	HAI titer of antibodies in hyperimmune sera of rats obtained to the indicated variants of the vaccine virus			
	Tex17	Tex17-8	Tex17-16	Tex17-17
Tex-wt	1280	320	640	640
Tex17	1280	320	320	1280
Tex17-8	2560	320	320	1280
Tex17-16	640	640	640	640
Tex17-17	1280	320	640	640

Table 5. Amino acid substitutions in surface antigens of experimental cellular monovalent LAIV

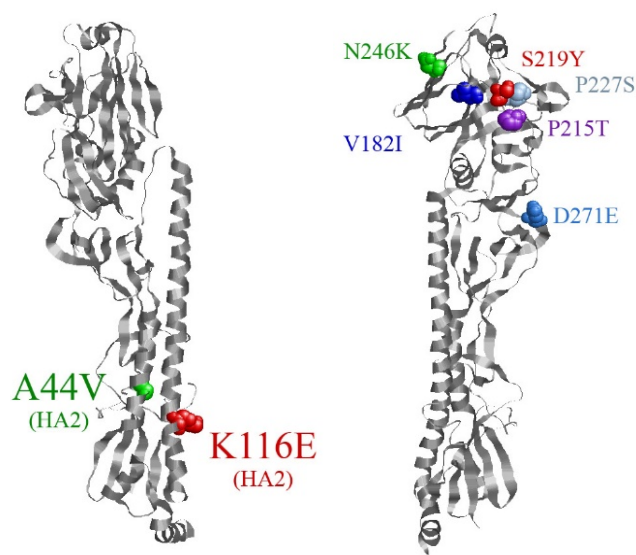| Vaccine	Gene	Protein	Amino acid	Original vaccine strain	Vaccine strain after production passages
A/17/Bolivia/2013/6585 (H1N1pdm)	HA	HA2	116	Lys	Glu
	NA	NA		Mutation not found	
A/17/Switzerland/2013/1 (H3N2)	HA	HA1	219	Ser	Tyr
			246	Asn	Lys
	NA	NA		Mutation not found	
B/60/Phuket/2013/26	HA	HA		Mutation not found	
	NA	NA		Mutation not found	

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Fig. 6. Mapping of adaptation amino acid substitutions in the HA molecule of the vaccine strains Calif17 and Bolivia17 (*a*), Tex17 and Switzerland17 (*b*).

The arrangement of amino acids is shown on the HA monomer of A/California/04/2009 (H1N1) and A/Singapore/H2011.447/2011 (H3N2) (PDBID: 3UYX and 4WEA, respectively). Illustrations were obtained using the «RasMol v. 2.7.5» program.

tivated influenza vaccine against H5N1 avian influenza. Passaging of viruses without additional conditions contributed to the emergence of adaptive mutations *N50K* (H1N1) and D160E (H3N2) in the HA2 molecule, which improved viral growth in cell culture, but negatively affected antigen stability [30]. H3N2 viruses grown in embryonated chicken eggs trigger conformational changes in the HA molecule under less acidic conditions than their antigenically similar primary isolates, and this phenotype was associated with the presence of amino acid substitutions *A138S*, *L194P* in HA1 and *D160N* in HA2, reduced resistance to acidic conditions and elevated temperatures [30]. Probably, the difference in pH levels during propagation in chicken embryos and cell culture caused the appearance of mutations in the region of the fusion peptide in HA2 in MDCK-adapted variants of the H3N2 vaccine strains Tex17-8, Tex17-16, Tex17-17 and H1N1 Calif17-8. Interestingly, that 5 mutations in the HA molecule were found only in the HA1 molecule, in particular, 4 of them (*A138S*, *G218R*, *P221L* and *V223I*) are located near the receptor-binding site [31]. The *N165K* mutation was localized in the HA antigenic site [31]. The *A138S* substitution can occur *in vivo* in immunocompromised patients as well as *in vitro* in MDCK cell culture and was found to cause a complete loss of α2,3-type receptor binding [32]. A mutation at position 218 in the HA1 molecule, which is located near the receptor-binding site, was also associated with decreased affinity for the human receptor and increased pathogenicity in a mouse model [33, 34]. The *L194P* amino acid substitution in HA1, which is prevalent in egg-adapted (passaged in embryonated chicken eggs

4–5 times) variants of H3N2 viruses, reduces binding and neutralization by broad-spectrum antibodies recognizing the receptor-binding site by 3 orders of magnitude, and also significantly changes the antigenicity of the HA molecule [35]. Despite the fact that some of the adaptation mutations detected in our study are located in significant places of the HA molecule, such as the receptor-binding site or antigenic sites, the described mutations do not affect the key parameters for the LAIV vaccine strain — the antigenicity of the MDCK-adapted strain and its immunogenicity do not change, but the replicative activity of the vaccine virus in cell culture increases, i.e., a useful property is acquired in the context of the production of cell-based LAIV. In this case, a decrease in infectious titers of MDCK-adapted LAIV strains in developing chicken embryos can be observed, which is an acceptable phenomenon when changing the substrate for culturing viruses. For the production of cultural LAIV, high replication activity in MDCK cell culture is most important, but not in eggs.

The influenza strain A/California/7/2009 (H1N1pdm) is genetically very similar to the A/California/4/2009 (H1N1pdm), but was isolated in the eggs and acquired the *Q226R* adaptation mutation during passaging, and after adaptation to mice, the *N159D* and *K212M* mutations were added. In this case, the virus with the *Q226R* mutation in HA1 lost affinity for human-type receptors and acquired affinity for avian receptors [36]. In both viruses, mutations located directly in the receptor-binding site expectedly affected the receptor specificity of the virus, disrupting the HA–NA balance, which led to a decrease in the reproductive activity of the virus in the MDCK-SIAT-1 cell culture. The mutations in the HA molecule that appeared after several more passages in mice became compensatory for the surface charge of the molecule and at the same time made a significant contribution to the increased pathogenicity of the studied viruses. Interestingly, data on the contribution of the *D225E* and *Q226R* mutations in HA to the virulence of the pandemic H1N1 influenza virus in mice were also obtained by Korean scientists [37]. In other studies, the *N159D* mutation is associated with escape mutations that allow the virus to evade the immune response [38].

During the preparation of the X-181 H1N1pdm09 vaccine strain in chicken embryos, the *N133D*, *K212T* and *Q226R* mutations were found in the HA1 molecule, which led to a strong change in the cross-reactivity of the vaccine strain, as a result of which the antibodies induced by the vaccine strain bound to the wild type of the virus, but did not neutralize it [39]. Evolutionary studies of the H1 molecule have shown the importance of amino acids at positions 156, 190 and 225 for adaptation to human receptors and antigenic drift of circulating H1N1 viruses [40].

Comparison of the H1N1pdm strains A/California/9/2007 and A/Brisbane/10/2010 revealed the pres-

ence of the *E47K* mutation in the HA2 molecule, which affected the stability of the HA trimer and decreased the threshold pH of membrane fusion from 5.4 to 5.0. It was also established that this occurs due to the presence of an intermonomer salt bridge between the amino acids *K47* in HA2 and *E21* in HA1 [41]. Furthermore, the *E47K* substitution increased the thermal stability of the virus and its virulence in ferrets, which indicates an advantage of the virus with *K47* in HA2 in adapting to evolutionary changes in humans [42]. The *V66H* mutation in HA2 at the site of contact between two subunits of the HA molecule of the influenza A/WSN/33 (H1N1) virus affects the shift of the maximum pH of membrane fusion towards lower values (from 5.6 to 5.1). In this case, the ability of the virus to replicate in MDCK was reduced, which was accompanied by a longer clearance of the virus in mice [43]. It can be noted that the adaptation mutations *A44V* in Calif17-8 and *K116E* in Bolivia17-M, detected in the present study, have a similar localization with the already described mutations *K47* in HA2 and *E21* in HA1. Probably, they can affect the threshold pH value during membrane fusion, which is the expected result of virus adaptation to a new substrate. Based on the data obtained, the adaptation of the influenza A(H3N2) vaccine virus to the MDCK cell culture led to the emergence of multiple amino acid substitutions in HA. It was shown that the *N154K* and *Y85E* mutations, detected in the HA2 subunit of the HA molecule of the vaccine strain, are critical for virus replication in culture. Furthermore, the *W92G*, *D160H* adaptation mutations in HA2 provide a significant advantage for the replication of the vaccine virus in the MDCK cells over its replication in the embryonated chicken eggs.

Comparison of the steric arrangement of adaptation mutations between strains adapted to MDCK cells in laboratory and industrial conditions revealed the similarity of these positions. Thus, in the H1N1 strain, amino acid substitutions *A44V* (Calif17-4) and *K116E* (Bolivia17) are in close proximity to each other in the HA2 subunit of the HA stalk-domain, and most of the adaptation substitutions found in H3N2 strains are in the globular domain of the HA1 molecule (Fig. 6). Thus, adaptation of LAIV vaccine strains to MDCK cells in laboratory and industrial conditions leads to the appearance of similar adaptation substitutions in strains of the same subtype, with influenza B viruses being maximally stable, and A/H1N1 viruses acquiring adaptation substitutions necessary for enhanced virus replication in this substrate. Strains A/H3N2 are the least stable, and adaptation mutations can also affect receptor-binding regions, which indicates the need for careful monitoring of the antigenic properties of strains of this subtype during their production on a cell substrate.

Conclusion

As a result of adaptation of the A/17/California/2009/38 (H1N1pdm09) vaccine strain to the MDCK cells, mutations *N156D* in HA1 and *A44V* in HA2 were found. A study of their effect on replicative activity *in vitro* showed that the presence of both mutations increases the titer of the vaccine strain in the MDCK cell culture by an order of magnitude, which gives advantages to this variant when it is produced on an industrial scale. The detected mutations retained the immunogenicity, cross-reactivity and protective efficacy of the MDCK-adapted strain Calif17-8 at the level of the egg-derived vaccine strain Calif17. Thus, the conducted studies show that the vaccine strain of LAIV subtype H1N1pdm09, obtained by the method of classical reassortment in developing chicken embryos, can be used for the production of LAIV on the MDCK cells.

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