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# The Effect of I155T, K156Q, K156E and N186K Mutations in Hemagglutinin on the Virulence and Reproduction of Influenza A/H5N1 Viruses

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**Abstract**—The continued circulation of influenza A virus subtype H5 may cause the emergence of new potential pandemic virus variants, which can be transmitted from person to person. The occurrence of such variants is mainly related to mutations in hemagglutinin (HA). Previously we discovered mutations in H5N1 influenza virus hemagglutinin, which contributes to virus immune evasion. The purpose of this work was to study the role of these mutations in changing other, non-antigenic properties of the virus and the possibility of their maintenance in the viral population. Mutations were introduced into the *HA* gene of a recombinant H5N1 influenza A virus (VNH5N1-PR8/CDC-RG) using site-specific mutagenesis. The "variant" viruses were investigated and compared with respect to replication kinetics in chicken embryos, thermostability, reproductive activity at different temperatures (33, 37 and 40°C), and virulence for mice. Amino acid substitutions I155T, K156Q, K156E+V138A, N186K led to a decrease in thermal stability, replication activity of the mutant viruses in chicken embryos, and virulence for mice, although these effects differed between the variants. The K156Q and N186K mutations reduced viral reproduction at elevated temperature (40°C). The analysis of the frequency of these mutations in natural isolates of H5N1 influenza viruses indicated that the K156E/Q and N186K mutations have little chance to gain a foothold during evolution, in contrast to the I155T mutation, which is the most responsible for antigenic drift. The A138V and N186K mutations seem to be adaptive in mammalian viruses.

**Keywords:** influenza A virus, H5 hemagglutinin, amino acid substitutions, site-specific mutagenesis, phenotypic properties, reverse genetics

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The highly pathogenic H5 avian flu viruses circulating in Asia, Africa and Europe in recent years contain the H5 hemagglutinin (*HA*) gene; its phylogenetic origin is the strain A/goose/Guangdong/1/96 (H5N1) (GsGD-lineage) isolated in 1996 during the epizootic in China [1]. Since that time, H5N1 influenza viruses have caused devastating epizootics among birds more than once, including the panzootic in 2005, which killed different species of birds and mammals, as well as humans. Single human cases of infection with the highly pathogenic H5N1 avian flu virus were recorded for the first time in China in 1997. In later years, the human cases and the number of infected individuals varied depending on epizootic situation in the region. According to the data of WHO for February 28, 2020, there were 861 laboratory-confirmed cases of infection in humans worldwide from 2003–2020, with a fatal outcome in 455 of these cases (https://www.who.int/influenza/human_animal_interface/2020_01_20_tableH5N1.pdf?ua=1).

The reassortment of genomes between the circulating influenza A viruses resulted in emergence of highly pathogenic strains with a modified set of genes but with the preserved H5 *HA* of the GsGD-lineage [2]. For example, the H5N8 influenza virus was isolated for the first time in 2010 from domestic ducks in China [3]. At present, H5N6 replaced H5N1 as the dominant subtype of highly pathogenic influenza viruses in South China, being spread mainly among domestic

ducks [4, 5] (https://www.oie.int/en/animal-health-in-the-world/update-on-avian-influenza/2020/).

Highly pathogenic H5Nx avian flu viruses are a serious hazard both for agriculture and for public health. The circulation of H5 avian flu viruses in the environment can lead to acquired mutations in these viruses, which may favor their distribution in the human population. At the moment, highly pathogenic H5 influenza viruses cannot be transmitted from person to person. However, if it happens, the world will be shaken by a new pandemic. This fact accounts for the unrelenting interest in H5 influenza viruses.

The object of our research is hemagglutinin (HA) of the H5N1 influenza virus. This is one of the glycosylated viral envelope proteins, which is responsible for the interaction with the cell receptor and for the fusion of viral and cellular membranes while the virus penetrates into the cell [6]. In addition, HA is the major antigenic determinant stimulating the immune response of infected organisms.

The rapid evolution of influenza viruses is determined by two processes. The first is the accumulation of mutations in viral proteins, and the second, the reassortment of genome segments between different strains. Mutations emerge spontaneously during viral genome replication due to errors in the viral RNA-dependent RNA polymerase, which lacks the proofreading ability. Under normal conditions, the emerging mutants are few and less viable than the parent strain. However, under certain conditions such as adaptation to a new host, immune response, or varying temperature regime, this diversity of mutants (quasispecies) becomes the starting material for the selection of viral variants more adapted to the new conditions [7].

In our previous studies of antigenic variability of H5N1 influenza viruses under immune pressure, we obtained viable mutants (escape mutants) with substitutions in the HA protein [8–10]. Some of these mutations can have a pleiotropic effect, i.e., in addition to modification of the antigenic properties, they influence the receptor specificity, virulence and thermal stability of the virus, which may contribute to effective distribution and extension of the host range of mutant variants and formation of viruses with a pandemic potential. Moreover, the screening of natural isolates of the H5 influenza virus HA with respect to positions of amino acid residues typical of escape mutants, which are responsible for antibody binding, has shown that some of these substitutions are present in viruses isolated not only from birds but also from humans and pigs. In this context, there is a need to investigate the effects of amino acid substitutions in such positions on different phenotypic characteristics of the virus in order to reveal their ability to gain a foothold in the viral population.

In the present work we have studied mutations in positions 138, 155, 156 and 186 of H5 HA (hereinafter, the numeration system is based on the H3 subtype [11]); previously we found them in escape mutants [9, 12] and Watanabe et al. [13] found them in the pathological material from patients infected by the H5N1 influenza virus. For elucidating the effects of the I155T, K156Q, K156E+V138A and N186K substitutions in HA on phenotypic traits by reverse genetics techniques, we have obtained VNH5N1-PR8/CDC-RG (H5N1) vaccine strain mutants characterized by substitutions in HA. The virulence, thermostability and temperature dependence of replication of the mutants have been studied.

## EXPERIMENTAL

**Reconstruction of the virus.** The original recombinant virus VNH5N1-PR8/CDC-RG (H5N1) and its mutants were obtained by reverse genetics. The virus was reconstructed using an eight-plasmid system kindly provided by Dr. R. Webster (St. Jude Children's Research Hospital, Memphis, United States) [14], two of them containing DNA copies of the *HA* and *NA* genes of the A/Vietnam/1203/2004 (H5N1) virus and the other containing 6 genome segments of the A/PuertoRico/8/34 (H1N1) influenza virus. In the system that we used, HA differs from the original A/Vietnam/1203/2004(H5N1) (GenBank, AY818135) by the A138V substitution and the shortened cutting site: QIETRG instead of QRERRRKKRG. The modification of the poly-based peptide in the cutting site decreases the high virulence typical of highly pathogenic H5N1 influenza viruses. Nucleotide substitutions required to obtain mutants were introduced with a commercial QuikChange XL Site-Directed Mutagenesis Kit (Stratagene, United States). The virus was assembled by plasmid transfection of the mixed HEK-293T–MDCK culture [14].

**Viruses** obtained by transfection were passaged once in 10-day chicken embryos through infection into allantoic cavity. Infected chicken embryos were incubated for 48 h at 37°C and then cooled overnight at 4°C. The virus-containing allantoic fluid was collected under sterile conditions and the content of the virus in the latter was determined by the titer in the hemagglutination reaction (RGA) [15] expressed in hemagglutinating units (HAU). The preparations were stored at –80°C.

**Polymerase chain reaction and sequencing.** Viral RNA was isolated from the virus-containing allantoic fluid with an RNeasy Mini Kit (Qiagen, Germany). Reverse transcription and PCR were performed using primers to the *HA* gene [16]. Amplification products were purified with a QIAquick PCR Purification Kit (Qiagen). Sanger sequencing was performed with a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, United States) and DNA ABI Prism 3130 automated DNA Sequencer (Applied Biosystems). Nucleotide sequences were analyzed using

the DNASTAR Sequence Analysis Software Package (DNASTAR Inc., United States).

**Determination of the infectivity of influenza viruses in chicken embryos.** To determine the 50% embryonic infection dose ($EID_{50}$), 10-day chicken embryos were infected with a 10-fold serial dilution of the virus introduced into the allantoic cavity (5 embryos per dilution). After 48-h incubation at 37°C, the infected chicken embryos were cooled overnight at 4°C and the presence of the virus in the allantoic fluid of each embryo was assessed in RHA with a 0.75% suspension of chicken red blood cells. The mean $EID_{50}$ for each virus was calculated using the Reed–Muench method (Reed & Muench) [17].

**Kinetics of accumulation of influenza viruses in chicken embryos.** The virus-containing allantoic fluid (1000 $EID_{50}$) infected 10-day chicken embryos (five embryos per each time interval) were incubated at 37°C for 18, 24, 36 and 48 h. After each time interval, the embryos were cooled overnight at 4°C and the content of the virus was determined in the allantoic fluid of each embryo using the RHA method.

**Reproductive activity of influenza viruses at different temperatures.** Ten-day chicken embryos were infected at a dose of 1000 $EID_{50}$/embryo (four embryos per each temperature point) and incubated in a thermostat (Binder, Germany) at 33, 37 and 40°C for 48 h. After the incubation, allantoic fluid was taken from each embryo to determine the content of the virus by the RHA method. The samples obtained at equal temperatures were used to prepare a combined sample by mixing equal volumes of the respective allantoic fluids. Combined samples were titered with respect to infectivity (see "Determination of infectivity of influenza virus in chicken embryos").

**Analysis of thermostability of hemagglutinin of influenza viruses.** The virus-containing allantoic fluid clarified by low-speed centrifugation was diluted with a phosphate buffered saline to 128 HAU and poured by 120 μL volumes into 10 0.5-mL thin-walled PCR tubes (SSI, United States), with 9 of them being thermostated in a Master-cycler Gradient 5331 thermal cycler (Eppendorf, Germany) at different temperatures within a range from 36.0 to 50.0°C for 40 min and then immediately placed in ice. The control sample was simultaneously stored (40 min) at 0°C. After the incubation, the virus titer in each sample was determined by the RHA method.

**Determination of pathogenicity of the influenza virus for mice.** The BALB/c mice, 8–10 g, were infected intranasally with 10-fold dilutions of each virus at doses from 1 to $10^6$ $EID_{50}$/mouse under light ether anesthesia. There were 5 mice in each group. The mice were observed for 16 days with examination, weighing, and count of surviving animals. The mice were qualified as "infected" if their weight decreased to 90% or below the initial value within 5 days. The 50% infective dose ($ID_{50}$) and 50% lethal dose ($LD_{50}$) for each virus were calculated by the Reed–Muench method (Reed & Muench) [17].

**Statistical data processing.** Statistical data analysis was performed using the parametric Student's test, the nonparametric Friedman test (ANOVA), and the Mann–Whitney U test. The critical significance level $p$ was taken as 0.05. The respective calculations were made using MS Office Excel 2016 and Statistica 8.0. The results are presented by the parameters of descriptive statistics: arithmetic mean value and standard deviation.

## RESULTS

### *Obtaining Mutant Influenza Viruses by Reverse Genetics*

Reverse genetics techniques were used to obtain 5 variants of the H5N1 influenza virus. The first variant, RGVN, is identical to the original vaccine strain VNH5N1-PR8/CDC-RG. The mutations causing amino acid substitutions in positions 155, 156, 138 and 186 of HA (I155T, K156Q, (K156E+V138A) and N186K, respectively) were introduced into the *HA* gene of the RGI155T, RGK156Q, RG(K156E+V138A) and RGN186K mutants by site-specific mutagenesis. We failed to obtain a mutant with a single K156E substitution. In the recombinant virus, this mutation was either absent or accompanied by an additional substitution: K156E+V138A or K156E+T160A. The structures of resultant mutants were confirmed by sequencing.

### *Kinetics of Accumulation of Influenza Viruses in Chicken Embryos*

The accumulation of influenza viruses in chicken embryos was assessed by the titer in RGA at 18, 24, 36 and 48 h after infection [11]. Almost equally high content of the virus in allantoic fluid was observed in all variants by hour 48 of the incubation, which apparently corresponded to the threshold accumulation in this cultivation system. For the RGI155T and RG(K156E+V138A) mutants, the kinetics of accumulation of the virus was not very different from the original RGVN virus. Accumulation of the RGK156Q and N186K viruses occurred with a lag, which was especially marked for the N186K mutant (Fig. 1). Thus, the I155T, K156Q, K156E+V138A and N186K amino acid substitutions in the H5N1 influenza virus had different effects on its replication activity in chicken embryos.

### *Thermostability of HA of Influenza Viruses*

The analysis of thermostability of genetically engineered mutants has shown differences in the temperature range of HA inactivation in the variants under study. In all tested mutants, thermostability was lower compared to the original RGVN virus. The RGN186K variants proved to be most sensitive to elevated tem-

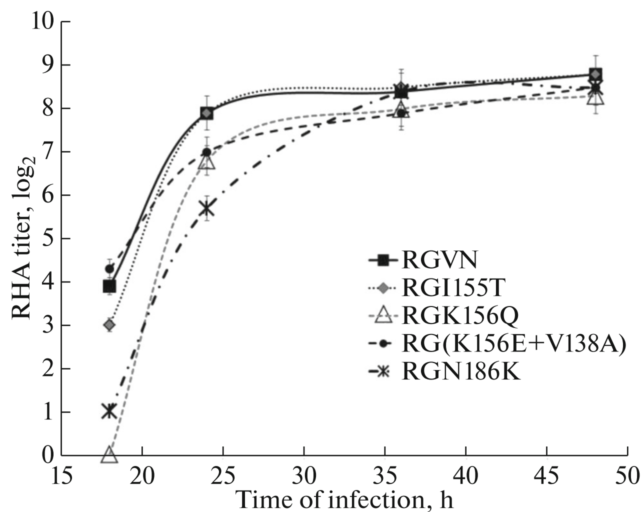


**Fig. 1.** The kinetics of accumulation of HA variants of the H5N1 influenza A virus in infected chicken embryos in multicycle infection. Here and on the next diagram, the average values with standard deviation calculated on the basis of three independent experiments are presented.

perature (Fig. 2). Thus, the study of hemagglutination activity of mutants of the RGVN influenza virus has shown that amino acid substitutions at positions 155, 156, (156 + 138) and 186 reduce the thermostability of HA of the H5N1 influenza virus.

*Reproductive Activity of Influenza Viruses at Different Temperatures (RCT)*

The randomized controlled trial (RCT) has shown that all variants under study (including the original variant) reproduce most effectively at a lower temperature (33°C). There are no statistically significant differences between the RGI155T and RG(K156E+V138A) viruses and the original RGVN virus in the level of replication at different temperatures (Table 1). At elevated temperatures, the titers of these variants decreased. A slight decrease in the infectious titer was observed for the RGK156Q and RGN186K mutants at 40°C (0.7 log$EID_{50}$) compared to the original RGVN virus. Thus, it can be asserted that the K156Q and N186K amino acid substitutions promote a decrease in reproductive activity of the H5N1virus at elevated temperatures (40°C).

*Virulence of Influenza Viruses for Mice*

The virulence of variants of the H5N1 influenza virus was assessed by variations in the weight of mice and their survival after intranasal infection at doses from 1 to $10^6$ $EID_{50}$/mouse. The $ID_{50}$ and $LD_{50}$ values for each virus were calculated on the basis of weight dynamics and survival rate, respectively. The original RGVN virus proved to be most virulent for mice; its $ID_{50}$ and $LD_{50}$ were similar: approximately $10^1$ $EID_{50}$/mouse (Table 2). The virulence of all mutant variants was lower (the higher the $LD_{50}$, the lower the virulence). For example, the virulence of the RGN186K strain decreased by 4 orders of magnitude; the K156Q substitution also resulted in $LD_{50}$ increase by almost 4 orders of magnitude. Such a great increase in $LD_{50}$ (i.e., reduction in lethality) of these two strains is probably due to the marked decrease in the ability to grow at 40°C and the lower thermostability of HA.

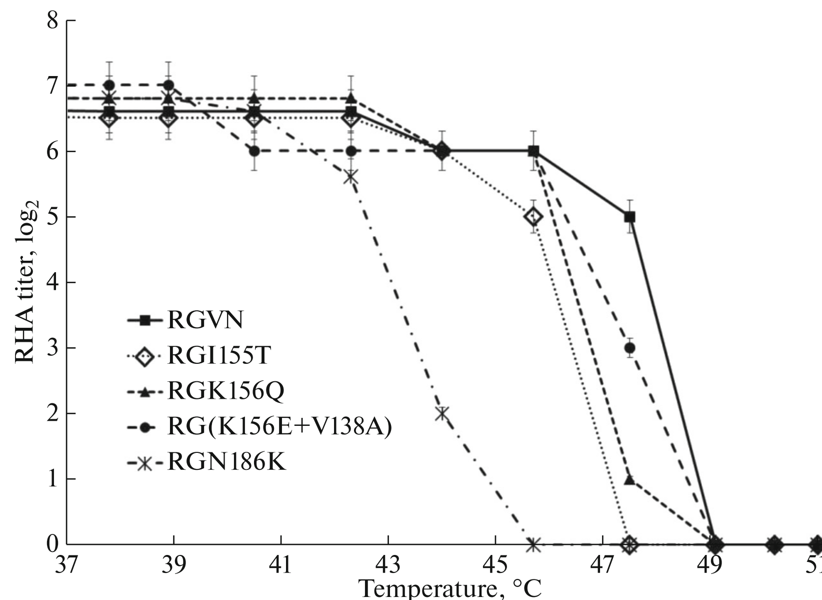


**Fig. 2.** The thermostability of HA of the studied variants of the H5N1 influenza A virus. The Y-axis is the titer of the virus after 40-min incubation at the indicated temperatures.

**Table 1.** Reproduction of variants of the H5N1 influenza A virus in chicken embryos at different temperatures [a]

| Virus | Amino acid substitution in HA | $\log EID_{50}$ | | |
|---|---|---|---|---|
| | | 33°C | 37°C | 40°C |
| RGVN | – | 8.20 ± 0.10 | 8.00 ± 0.20 | 7.50 ± 0.20 |
| RGI155T | I155T | 8.65 ± 0.15 | 8.20 ± 0.20 | 7.20 ± 0.20 |
| RGK156Q | K156Q | 7.75 ± 0.25 | 7.00 ± 0.25 | 6.80 ± 0.20 |
| RG(K156E+V138A) | K156E+V138A | 8.65 ± 0.15 | 8.20 ± 0.20 | 7.55 ± 0.15 |
| RGN186K | N186K | 8.10 ± 0.20 | 7.75 ± 0.15 | 6.80 ± 0.20 |

[a]The data are presented as an infectious titer of the virus 48 h after the infection of chicken embryos. The results are given as the mean ± SD calculated on the basis of three independent experiments.

**Table 2.** The effects of amino acid substitutions in HA of the RGVN influenza virus on pathogenicity for mice[a]

| Virus | Amino acid substitution in HA | $\log ID_{50}$ | $\log LD_{50}$ |
|---|---|---|---|
| RGVN | – | 0.90 ± 0.20 | 1.10 ± 0.10 |
| RGI155T | I155T | 2.00 ± 0.30 | 2.50 ± 0.20 |
| RGK156Q | K156Q | 1.00 ± 0.30 | 4.00 ± 1.00 |
| RG(K156E+V138A) | K156E+V138A | 1.00 ± 0.20 | 2.50 ± 0.40 |
| RGN186K | N186K | 4.00 ± 0.30 | 4.30 ± 0.40 |

[a]The results are presented as the mean ± SD calculated on the basis of two experiments.

### *Screening of the H5 HA Variants of Influenza Viruses Circulating in Nature*

The frequency of occurrence of amino acid substitutions under study in the natural isolates of H5 influenza viruses was estimated by HA screening of the H5 influenza viruses over the past 17 years according to the GenBank (https://www.ncbi.nlm.nih.gov/genomes/FLU/Database/nph-select.cgi#mainform) and GISAID EpiFlu (https://platform.gisaid.org) databases (Table 3).

## DISCUSSION

Previously we studied the effects of amino acid substitutions in the HA molecule of the H5 influenza virus on the phenotypic traits of escape mutants (resistant to the neutralizing effect of any monoclonal antibody) [12, 18]. One of manifestations of the evolutionary variability of highly pathogenic H5N1 influenza viruses during their adaptation to a new host and/or reproduction conditions is associated with HA changes in antigenic sites, receptor-binding sites (RBS), HA glycosylation, and cutting[1] sites [19]. On the threshold of a possible pandemic caused by the H5 influenza viruses, the efforts of scientists are focused on the search of mutations that may be genetic markers of adaptation of these viruses to mammals and their spread among humans.

[1] The cutting or cleavage of HA on the HA1 and HA2 chains is necessary for the activation of the infectious process in cells.

The pathological material from patients infected with the H5N1 influenza virus was shown to contain mutant HA variants (quasispecies) with the following as the most frequently occurring mutations: A138V, N186D, S227N, I155T, K156Q, and N186K [13, 20]. Previously we have detected mutations in these positions in escape mutants obtained under exposure to monoclonal antibodies and in readaptants obtained by passaging low-virulence escape mutants in the lungs of mice [9, 12, 21].

In this work we used site-specific mutagenesis to obtain viral variants with the I155T, K156Q, K156E+V138A and N186K substitutions in H5 HA, with the same structure of other genes, and have shown that phenotypic changes in these variants are due to single mutations in HA.

In a three-dimensional H5 HA molecule, the amino acids in positions 155 and 156 are located in antigenic site 1, which corresponds to antigenic site B of the H3 HA molecule [22]. Next to it there is RBS, a depression in the globular part of HA, the so-called "receptor pocket". The amino acids in positions 138, 155 and 186 are on the surface of the HA molecule and flank the receptor pocket on different sides. Amino acid 155 in the boundary region simultaneously forms part of both the antigenic site and RBS [23]. Amino acids 138V and 186K adjoining RBS reduce the binding of H5N1 viruses to avian-type cell receptors (Neu5Ac$\alpha$2-3Gal) and increase the affinity to human-type receptors (Neu5Ac$\alpha$2-6Gal) and thus

**Table 3.** The frequency of occurrence of amino acid substitutions in H5 HA among the natural influenza viruses isolated from 2003 to 2019

| Year of isolation | Amino acid substitution, %[a] | | | | |
|---|---|---|---|---|---|
| | I155T | K156Q | K156E | A138V[b] | N186K |
| 2003 | **0** (0/137) | **0** (0/137) | **0.7** (1/137) | **0.7** (1/137) | **0** (0/137) |
| 2004 | **1.2** (6/489) | **0.4** (2/489) | **0.6** (3/489) | **1.0** (5/489) | **0.2** (1/489) |
| 2005 | **0.5** (4/749) | **0.1** (1/749) | **0** (0/749) | **1.5** (11/749) | **0.5** (4/749) |
| 2006 | **1.4** (18/1326) | **0.1** (1/1326) | **0** (0/1326) | **0.7** (9/1326) | **0.3** (4/1326) |
| 2007 | **2.5** (24/948) | **0.5** (5/948) | **0** (0/948) | **0.6** (6/948) | **0** (0/948) |
| 2008 | **4.8** (27/561) | **1.2** (7/561) | **0.7** (4/561) | **0.9** (5/561) | **0.2** (1/561) |
| 2009 | **16.4** (89/542) | **0.2** (1/542) | **1.8** (10/542) | **1.1** (6/542) | **0** (0/542) |
| 2010 | **17.9** (114/638) | **0.2** (1/638) | **1.1** (7/638) | **0.6** (4/638) | **0** (0/638) |
| 2011 | **22.2** (140/631) | **0** (0/631) | **0** (0/631) | **0.3** (2/631) | **0.3** (2/631) |
| 2012 | **8.5** (35/410) | **1.0** (4/410) | **0** (0/410) | **0.0** (0/410) | **0** (0/410) |
| 2013 | **17.7** (76/430) | **0** (0/430) | **0.9** (4/430) | **0.2** (1/430) | **0** (0/430) |
| 2014 | **26.8** (219/818) | **0.1** (1/818) | **0.1** (1/818) | **0.1** (1/818) | **0** (0/818) |
| 2015 | **43.6** (682/1564) | **0** (0/1564) | **0** (0/1564) | **0.2** (3/1564) | **0** (0/1564) |
| 2016 | **46.5** (348/748) | **0** (0/748) | **0** (0/748) | **0.0** (0/748) | **0.1** (1/748) |
| 2017 | **19.6** (113/576) | **0** (0/576) | **0** (0/576) | **0.0** (0/576) | **0** (0/576) |
| 2018 | **10.8** (24/222) | **0.5** (1/222) | **0** (0/222) | **0.5** (1/222) | **0** (0/222) |
| 2019 | **17.6** (3/17) | **0** (0/17) | **0** (0/17) | **0.0** (0/17) | **0** (0/17) |

[a]The number of sequences with the indicated substitution/total number of H5 viruses within the specified period is given in brackets. [b]This amino acid substitution is present in the original RGVN virus.

can be considered to be associated with adaptation [13, 20, 24–27] (Fig. 3).

It should be noted that the overwhelming majority of H5 influenza viruses (8514 HA sequences) have an alanine residue in position 138, and only 12 natural isolates of the highly pathogenic H5N1 line contain the A138V substitution. Among them, 11 viruses were isolated from people during the period from 2004 to 2010, which confirms that the A138V mutation is associated with adaptation [20]. The same A138V mutation is present in all the RG mutants that we studied, except for RG(K156E+V138A), where the concomitant V138A mutation emerged as a return to the wild type. In all likelihood, this was not accidental and explains all our unsuccessful attempts to obtain a RG mutant with a single K156E substitution. Previously we revealed a single K156E mutation only in escape mutants of the VNH5N1-PR8/CDC-RG virus with HA containing an alanine residue (A138) in position 138 [9]. It should be added that Auewarakul et al. [26] failed to reconstruct the influenza virus with a single A138V mutation in HA from A/Thailand/676/2005 (H5N1) and other genes from PR8 (H1N1) by reverse genetics, which they believed was due to incompatibility between this mutation and other genes. In the mutants obtained, the A138V substitution was always accompanied by an additional mutation in HA.

According to Naughtin et al. [27], influenza viruses with the A138V mutation in HA reproduce well in chicken embryos and in the MDCK mammalian cell culture. In all likelihood, the initial RGVN virus containing an adaptation-associated mutation A138V has a well-balanced structure, which allows it to successfully reproduce in chicken embryos. The introduced K156Q, K156E+V138A and N186K mutations disturb this balance as is evidenced by the decrease in the virus titer in the first 24 h after the infection of chicken embryos (Fig. 1). In this context, it is significant that there is a considerable delay of growth of the RGN186K mutant carrying, in addition to 138V, the N186K substitution that switches the receptor specificity of HA from “avian” to “human”.

The revealed decrease in virus reproduction at a higher (40°C) temperature and the thermostability of HA of the RGN186K mutant is in agreement with the data of other authors showing, in addition to these properties, that a single N186K mutation results in a higher affinity to “human”-type receptors, enhanced replication in the culture of epithelial cells of the human respiratory tract, and increased pH (from 5.6 to 5.9) of activation of conformational changes in HA (the parameter essential for penetration of the virus into the cell) [13, 25]. All these characteristics suggest that the N186K substitution can promote the transfer of H5N1 influenza viruses to a new host (mammals).

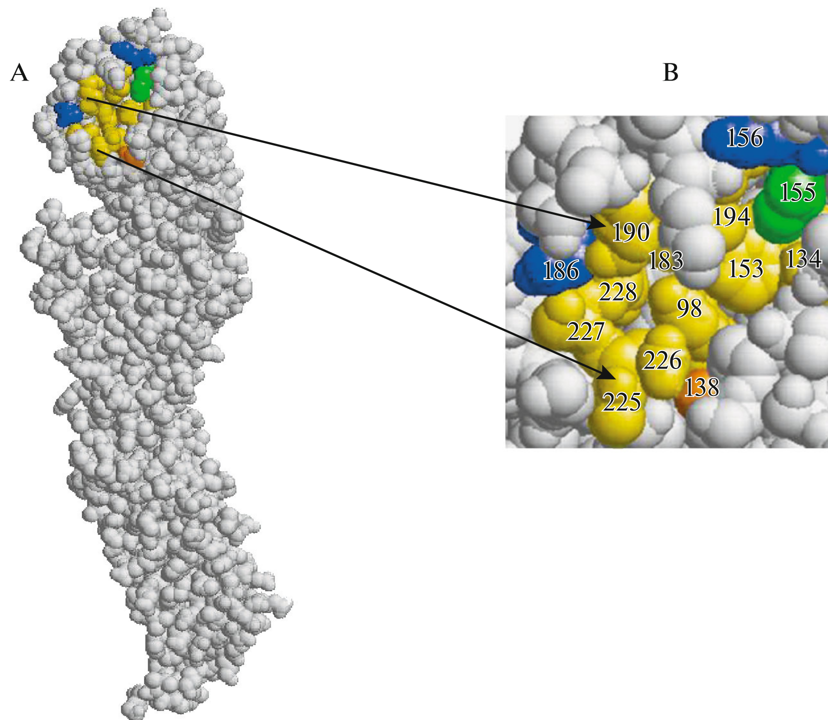


**Fig. 3.** The positions of the amino acid substitutions in the three-dimensional structure of the A/Vietnam/1203/2004 influenza virus (according to [28]). The positions of amino acid substitutions (in the H3 numeration) are blue for the antigenic site B, yellow for RBS, and green for site B and RBS simultaneously. Position 138 is in orange. The image was obtained with RasMol (www.rasmol.org); the structure of H5 molecule was taken from the RCSB Protein Data Bank (PDB ID 2FKO) ([DOI] (https://doi.org/https:doi.org/10.2210/pdb2FKO/pdb).

However, this event most likely requires some additional conditions, which confirms the avirulence of the RGN186K variant for mice compared to the initial RGVN virus (Table 2) and the extremely low frequency of occurrence of this mutation (Table 3).

The K156Q amino acid substitution, similar to the N186K mutation, resulted in lower HA thermostability and reproduction at an elevated temperature (40°C) (Fig. 2, Table 1), and attenuation of virulence for mice (Table 2). Position 156 in the antigenic site is characterized by polymorphism (there are 10 known variants of amino acid residues) and is responsible for the antigenic variability of HA. The replication activity and virulence of the influenza virus depend on a particular amino acid in this position and on the nature of the virus [29–31]. The K156Q mutation was found in 24 natural H5N1 influenza viruses isolated mainly from birds. The earliest strain with this substitution, A/duck/Shantou/195/2001(H5N1), was isolated from domestic ducks in China and is considered to be one of the first precursors of the highly pathogenic strains of the H5N1 influenza virus that caused epizootics in Southeast Asia in 2003 [32]. Later on (2004–2012), the K156Q mutation was observed in the isolates from chickens in Thailand, Indonesia and India and from crows (India, 2012), as well as in two isolates from humans in Egypt (2008 and 2009) and from pigs in China (2014). The viruses isolated in different countries belong to different clades of the GsGD-lineage, i.e., only 3 cases of the presence of this mutation in mammalian influenza viruses have been recorded over the past 20 years of evolution of the H5 influenza viruses. It is unlikely that this mutation promotes the transfer of this virus to mammals.

The K156E substitution leads to a change in the amino acid charge from positive to negative, thereby weakening the electrostatic interaction between the virus and the cell surface and contributing to more effective distribution of progeny viruses [29–31]. The RG(K156E+V138A) mutant successfully reproduced in chicken embryos within the entire range of temperatures under study, though its thermostability was lower compared to the initial RGVN virus (Table 1); at the same time, virulence was lower compared to RGVN but remained at a rather high level for mice. The combination of 156E+138V substitutions has not been found in natural isolates, which partially explains our failure to create a viable recombinant mutant with this combination. The 156E+138A combination, like in the RG(K156E+V138A) mutant, has been found in 18 natural isolates from birds, with 12 viruses belonging to the GsGD-lineage and the rest of them being nonpathogenic influenza viruses from the American continent and South Africa.

The I155T mutation leads to a decrease in HA thermostability and attenuation of virulence of the virus for mice but has no significant effect on viral reproduction in the temperature range under study compared to the initial virus (Fig. 2, Table 1). The I155T substitution is widespread in nature among the viruses of domestic and wild birds, as well as humans. The emergence and fixation of this mutation are most likely determined by selection under the conditions of immune pressure. One of the first cases of mentioning (without comments) this mutation in "human" H5 influenza viruses can be found in the work by Kongchanagul et al. [20]. In the study of HA quasispecies diversity in different organs of patients who fell victims to the H5N1 avian flu in Thailand in 2004–2005, the I155T mutation was observed only once in viral material from a patient's lungs on day 17 of the disease. Hence, it can be supposed that the mutation emerged under the pressure of immune response formed at the late stages of the disease (our comments). Over the past 10 years (2010–2020), the frequency of occurrence of the I155T substitution in HA of influenza viruses isolated from humans is 48.5%. Incidentally, this mutation is present in the sample isolated from a human in China in 2018 (GenBank, MK300699).

Generalization of research data on single mutations in HA leads to the conclusion that the mutations that alter the antigenic properties of the H5 influenza A virus may have a pleiotropic effect. The A138V mutation provides the high virulence, thermostability and enhanced reproduction of the virus in the temperature range from 33 to 40°C. The I155T and K156E+V138A mutations contribute to viral reproduction at the lower temperature (33°C) with the maintenance of high virulence. The K156Q and N186K mutations lead to attenuation of virulence and a decrease in the reproductive ability of the influenza virus at 40°C.

The analysis of the frequency of occurrence of these mutations in natural isolates indicates that the K156E/Q and N186K mutations have little chance of being preserved during evolution, in contrast to the I155T mutation, which is maximally responsible for the antigenic drift of HA. The A138V and N186K mutations are associated with adaptation in mammalian influenza viruses.

In all likelihood, the formation of new viable variants of the H5 influenza A viruses with prolonged circulation requires not single mutations in HA but most likely a particular combination of several mutations in HA and/or other viral proteins.

## COMPLIANCE WITH ETHICAL STANDARDS

*Conflict of interests*. The authors declare that they have no conflict of interest.

*Statement on the welfare of animals*. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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