Russian Journal of Infection and Immunity = Infektsiya i immunitet
2022, vol. 12, no. 5, pp. 837–849

Инфекция и иммунитет
2022, Т. 12, № 5, с. 837–849

REDUCED AMINO ACID ALPHABET-BASED ENCODING AND ITS IMPACT ON MODELING INFLUENZA ANTIGENIC EVOLUTION

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Abstract. Currently, vaccination is one of the most efficient ways to control and prevent influenza infection. Vaccine production largely relies on the results of laboratory assays, including hemagglutination inhibition and microneutralization assays, which are time-consuming and laborious. Viruses can escape from the immune response that results in the need to revise and update vaccines biannually. The hemagglutination inhibition assay can measure how effectively antibodies against a reference strain bind and block an antigen of the test strain. Various computer-aided models have been developed to optimize candidate vaccine strain selection. A general problem in modeling of antigenic evolution is the representation of genetic sequences for input into the research model. Our motivation stems from the well-known problem of encoding genetic information for modeling antigenic evolution. This paper introduces a two-fold encoding approach based on reduced amino acid alphabet and amino acid index databases called AAindex. We propose to apply a simplified amino acid alphabet in modeling of antigenic evolution. A simplified alphabet, also called a sub-alphabet or reduced amino acid alphabet, implies to use the 20 amino acids being clustered and divided into amino acid groups. The proposed encoding allows to redefine mutations termed for amino acid groups located in reduced alphabets. We investigated 40 reduced amino acid sets and their performance in modeling antigenic evolution. The experimental results indicate that the proposed reduced amino acid alphabets can achieve the performance of the standard alphabet in its accuracy. Moreover, these alphabets provide deeper insight into various aspects of the relationship between mutation and antigenic variation. By checking identified high-impact sites in the Influenza Research Database, we found that not only antigenic sites have a significant influence on antigenicity, but also other amino acids located in close proximity. The results indicate that all selected non-antigenic sites are related to immune responses. According to the Influenza Research Database, these have been experimentally determined to be T-cell epitopes, B-cell epitopes, and MHC-binding epitopes of different classes. This highlighted a caveat: while simulating antigenic evolution, the model should consider not only the genetic information on antigenic sites, but also that of neighboring positions, as they may indirectly impact antigenicity. Additionally, our findings indicate that structural and charge characteristics are the most beneficial in modeling antigenic evolution, which is in agreement with previous studies.

Key words: *AAindex, antigenic evolution, hemagglutinin, influenza, modeling, reduced amino acid alphabet.*

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Для цитирования:
Форгани М., Фирстков А.Л., Аляннеджади М.М., Даниленко Д.М., Комиссаров А.Б. Кодирование с помощью сокращенного аминокислотного алфавита и его влияние на моделирование антигенной эволюции гриппа // Инфекция и иммунитет. 2022. Т. 12, № 5. С. 837–849. doi: 10.15789/2220-7619-RAA-1968

Citation:
Forghani M., Firstkov A.L., Alyannezhadi M.M., Danilenko D.M., Komissarov A.B. Reduced amino acid alphabet-based encoding and its impact on modeling influenza antigenic evolution // Russian Journal of Infection and Immunity = Infektsiya i immunitet, 2022, vol. 12, no. 5, pp. 837–849. doi: 10.15789/2220-7619-RAA-1968

This study was funded by the Russian Foundation for Basic Research (RFBR), project number 19-31-60025.

DOI: http://dx.doi.org/10.15789/2220-7619-RAA-1968

КОДИРОВАНИЕ С ПОМОЩЬЮ СОКРАЩЕННОГО АМИНОКИСЛОТНОГО АЛФАВИТА И ЕГО ВЛИЯНИЕ НА МОДЕЛИРОВАНИЕ АНТИГЕННОЙ ЭВОЛЮЦИИ ГРИППА

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Резюме. В настоящее время, вакцинация является одним из наиболее эффективных способов контроля и профилактики гриппозной инфекции. Производство вакцин в основном зависит от результатов лабораторных анализов, включая анализ реакции торможения гемагглютинации и микронейтрализации, которые требуют много времени и труда. Вирусы могут избегать иммунного ответа, что приводит к необходимости пересмотра и обновления вакцин два раза в год. Анализ реакции торможения гемагглютинации позволяет измерить, насколько эффективно антитела против эталонного штамма связывают и блокируют антиген испытуемого штамма. Для оптимизации выбора вакцинного штамма-кандидата были разработаны различные компьютерные модели. Одной из общих проблем в моделировании антигенной эволюции является представление генетических последовательностей для ввода в исследовательскую модель. Наша мотивация связана с хорошо известной проблемой кодирования генетической информации для моделирования антигенной эволюции. В данной работе представлен двухэтапный подход к кодированию, основанный на сокращенных аминокислотных алфавитах и базах данных аминокислотных индексов под названием AAindex. Мы предлагаем использовать упрощенные аминокислотные алфавиты для моделирования антигенной эволюции. Упрощенный алфавит, также называемый субалфавитом или сокращенным аминокислотным алфавитом, это алфавит, в котором 20 аминокислот разделены на группы. Предложенное кодирование позволяет переопределить мутации в терминах групп аминокислот, расположенных в сокращенном алфавите. Мы исследовали 40 сокращенных алфавитов и их эффективность при моделировании антигенной эволюции. Результаты экспериментов показывают, что предложенные сокращенные аминокислотные алфавиты могут достичь показателей стандартного алфавита по точности. Более того, эти алфавиты позволяют лучше понять взаимосвязь между мутациями и антигенными изменениями с различных точек зрения. Проверив полученные высокоэффективные сайты в исследовательской базе данных гриппа (Influenza Research Database), мы обнаружили, что не только антигенные сайты оказывают значительное влияние на антигенность, но и их соседние аминокислоты. Результаты показывают, что все выбранные неантигенные участки связаны с иммунным ответом. Согласно исследовательской базе данных гриппа, экспериментально установлено, что это эпитопы Т-клеток, эпитопы В-клеток и MHC-связывающие эпитопы различных классов. Это подчеркивает значимость того, что: при моделировании антигенной эволюции модель должна учитывать не только генетическую информацию антигенных участков, но и генетическую информацию соседних позиций, поскольку они могут косвенно влиять на антигенность. Кроме того, наши результаты показывают, что, в соответствии с предыдущими исследованиями, структурные и зарядовые характеристики аминокислот являются наиболее значимыми при моделировании антигенной эволюции.

Ключевые слова: *AAindex, антигенная эволюция, гемагглютинин, грипп, моделирование, сокращенный аминокислотный алфавит.*

Introduction

Influenza is a contagious respiratory infection that affects 5–15% of the population worldwide annually, resulting in 3–5 million cases of severe illness and 250 000 to 500 000 deaths [36]. Influenza epidemics influence public health and involve severe economic consequences, making it the subject of various economic studies [4]. The World Health Organization (WHO) continuously monitors viral pathogens, especially those that can become epidemics or pandemics, and decides on strategies to combat them. Given the special status of influenza, the WHO created the Global Influenza Surveillance and Response System, the primary function of which is to monitor the evolution of the influenza virus and to provide recommendations for the annual vaccine's composition for the Northern and Southern Hemispheres.

Influenza viruses are part of the *Orthomyxoviridae* family. According to antigenic characteristics of their nuclear proteins, they are grouped into four types: IVA (A); IVB (B); IVC (C); and IVD (D). Among them, types A and B are associated with influenza outbreaks. Type C appears to evolve slowly and leads to less severe and less significant health consequences. Type D is an influenza C-like virus that is observed in non-human hosts, e.g., cattle and swine [30]. Type A is further classified according to the combination of hemagglutinin (HA) and neuraminidase (NA), the two main surface antigens of influenza that play a key role in infectivity and

immune responses. HA has 18 variants (H1–H18), while the NA protein can be one of 11 variants (N1–N11). Hence, the virus can theoretically be any of 198 different subtypes; this provides an ability to infect a broad spectrum of various hosts [37]. Despite this diversity, humans are infected with only a limited number of influenza A subtypes (i.e., H1N1, H2N2, H3N2), with H1N1 and H3N2 being currently relevant. Thus, we consider them in this paper. Other zoonotic subtypes represent only sporadic infections and are out of the scope of this study.

Influenza A viruses are capable of enormous genetic variation, both through continuous, gradual mutation and by reassortment of gene segments between viruses, resulting in emerging novel antigenic variants. Epidemics are the result of gradual evolutionary changes called antigenic drift, which leads to the generation of new strains from existing ones through mutation. In addition to antigenic drift, the influenza virus can be altered by antigenic shift. It is an abrupt significant change in influenza viruses resulting in the emergence of new HA and/or NA. It is the process by which at least two subtypes combine into a new subtype that has a mixture of surface antigens of two or more strains [35].

The only effective method to control influenza is vaccination, eliciting protective neutralizing antibodies and memory T-cell responses. Since HA antigen abundance on the viral surface is approximately four-fold greater than NA [31], it is the primary component in vaccine compositions. This is the reason why we consider only HA protein sequences in this paper.

The influenza vaccine requires an update if the vaccine composition strains are antigenically distinct from currently circulating viruses. A gold-standard and widespread laboratory procedure called hemagglutination inhibition (HI) assay is used to assess the measure of antigenic similarity between strains. The HI assay can measure how effectively antibodies against a reference strain bind and block an antigen of the test strain. High HI titers indicate a high degree of antigenic similarity between strains [16]. The main conclusion of HI assay analysis is determining antigenic distance (i.e., similarity between reference and test antigens), which further can be presented in terms of a binary variable called antigenic variant. Currently, there are two widely used definitions of antigenic distance [18, 27]:

$$d_1(i,j) = log_2(\frac{M}{H_{i,j}}) \quad (1)$$

$$d_2(i,j) = \sqrt{\frac{H_{i,i} \times H_{j,j}}{H_{i,j} \times H_{j,i}}} \quad (2)$$

where $H_{i,j}$ is the obtained HI titer for antiserum of (reference) strain j against the antigen of (test) strain i, and M is the maximum titer observed for antiserum j against any antigen in the HI table. The antigenic variant is determined by applying the threshold to the obtained antigenic distance. The pair of test and reference viruses whose antigenic distance meets the threshold are designated as antigenic variants; otherwise, they are only antigenically similar.

The HI assay is a labor-intensive and time-consuming procedure, while vaccine development is under time pressure. Over the past decade, various computer-aided approaches have been developed to speed up the process of strain selection and to increase the quality of vaccine production. Klingten et al. [16] has provided a comprehensive review of antigenic evolution prediction associated with vaccine production. They classified the approaches into phylogenetic and population genetics-based methods, statistical methods, epidemiological models, and other methods based on information and graph theories. The approaches employ different data types serving as model inputs, e.g., viral sequence, HI assay data, protein structure, physicochemical properties, etc. A critical step in antigenic variant modeling is describing the biological significance of a mutation between test and reference viruses and linking it to antigenicity.

Unfortunately, the exact roles and how they affect biological properties within evolution are not yet fully understood for many such changes. Generally, it is known that evolution is influenced by several biological properties, especially the volume and hydrophobicity of amino acids [32]. Studies on amino acid property changes provide fundamental information about the evolution of specific proteins. Earlier studies indicated that HA antigen is positively charged, while on the contrary, the glycan receptors of the host cell are negatively charged. Thus, changes in electrostatic charge due to mutation can play a significant role in receptor specificity, enhancing or diminishing the receptor binding affinity and avidity [2, 17]. Moreover, Huang et al. [14] recently showed that charged amino acid mutations impact influenza virus evolution and are beneficial in vaccine research. Accordingly, mutation can be considered a multidimensional event, wherein each dimension represents an amino acid attribute.

Several techniques reflect the biological characteristics of mutation in numerical domains, among which application of the AAindex database [15] is the most popular. The AAindex database is a comprehensive collection of biological, physical, and chemical amino acid properties collected from scientific papers and accessed through www.genome.jp. The database mainly consists of three sections: AAindex1; AAindex2; and AAindex3. AAindex1 includes various amino acid indices, each of which can be represented as a numerical vector of 20 numbers representing 20 standard amino acids. AAindex2 contains different amino acid mutation matrices, while AAindex3 consists of statistical protein con-

tact potentials. The AAindex database (ver.9.2) currently covers 566, 94, and 47 records for AAindex1, AAindex2, and AAindex3, respectively.

As mentioned, the AAindex database has been employed for encoding protein sequence in various studies. Here, we mention some of the more relevant studies in which the AAindex database was used for exploring genetic and antigenic evolution. Yao et al. [39] proposed an algorithm called joint random forest regression to predict antigenic variants. They compared 95 amino acid matrices, including AAindex2, to assess the relationship between genetic and antigenic evolution by amino acid attributes at different protein sites. Their results indicated that structural features are more significant to the antigenicity of the influenza virus. Wang et al. [34] suggested an approach based on matrix completion for predicting antigenic evolution. They studied the impact of 65 amino acid substitution matrices taken from the AAindex database to predict antigenic evolution. Their results suggested that the "homologous structure derived matrix (called HSDM) for alignment of distantly related sequences" outperformed others in terms of RMSE.

Moreover, Qiu et al. [24] developed a structure-based antigenicity scoring model. Their model engages antigenically dominant positions according to structural context, including correlation with local amino acid attribute changes, to analyze antigenicity. They demonstrated that incorporating the structural context of protein can enhance antigenic evolution prediction. Additionally, Forghani and Khachay [10] carried out a principal component analysis on AAindex1 and introduced 11 indices that explained 91% of the total variation in the database. The new indices are further used to encode HA protein sequence and create an input tensor fed into a convolutional neural network. Their model achieves a mean absolute error of 0.935 antigenic units for yearly, non-anticipating prediction of antigenic distance for subtype H1N1 (2001–2009). Cui et al. [6] suggested modeling influenza virus antigenicity by selecting the most significant sites, clustering the AAindex1 based on mutual information, and encoding the sites by the representative from clusters to form the feature vector. The feature vector is further given to a classifier to discretize antigenic variant classes. Recently, we performed a preliminary analysis to study the impact of amino acid encoding on modeling the antigenic evolution of the influenza virus [11]. Apart from Cui et al.'s work, our work introduces an early-stage mutation encoding by applying reduced amino acid alphabets.

The current paper addresses one of the fundamental challenges in bioinformatics: deciding how to represent input genetic information for modeling more efficiently and meaningfully. In response to this problem, we employed simplified amino acid alphabets. A simplified alphabet, also called a sub-alphabet or reduced amino acid alphabet (RAAA), is an alphabet in which the 20 amino acids are clustered and divided into amino acids groups. RAAA construction is a problem that belongs to the set partitioning problem, which is out of this paper's scope. Previous studies have shown that RAAAs have been successfully applied in various domains, including: protein annotation and description; protein functionality prediction [21, 41]; protein folding assessment; sequence classification [19]; consensus sequence search; and genetic pattern identification [5].

A reduced amino acid set simplifies protein system complexity, providing a better insight into structural similarities across protein sequences [42]. We considered different definitions of similarity via RAAAs to reconstruct the relationship between genotype and phenotype. A RAAA represents genetic information on a coarse scale, which may highlight attributes that drive antigenic evolution of the influenza virus.

In our approach, encoding is conducted in two steps. In addition to the standard amino acid alphabet, the first step employs a RAAA to represent the mutation in different structural, biological, and physicochemical contexts. Further, the second step encodes the alphabetical information of the encoded genetic sequence into a numerical one, which enables its use in various types of mathematical modeling. Preliminary results indicate that some RAAA-based models outperform models based on the standard amino acid alphabet in terms of accuracy.

In this paper, we take a step forward and perform a comprehensive analysis to further refine result accuracy. The contributions of this paper are three-fold:

1. We propose a novel encoding method using reduced amino acid alphabets, which helps to clarify the genetic/antigenic relationship.

2. Relative to similar previous studies [6, 11], we improve the approach at several levels:

   2.1. Increasing the resolution of thresholds.

   2.2. Clustering by several methods and comparing their results to find the optimal number of clusters.

   2.3. Selecting the closest index to the center of a cluster as its representative.

   2.4. Applying five well-known classification algorithms.

   2.5. Optimizing of classifier hyperparameters through a comprehensive grid search.

3. Relying on experimental results, we found that incorporating structural and charge properties can enhance modeling quality, which is in agreement with previous studies.

The rest of the paper is organized as follows. Section "Materials and methods" describes the general computational pipeline, data preparation, and all necessary algorithms for primary and secondary encoding. Experimental setup and its outcomes are presented in Section "Results and discussion". This section also covers interpretation and discussion of the obtained results. Finally, the results of our study are summarized in Section "Conclusion".

Materials and methods

As mentioned earlier, our experimental design was inspired by a published methodology [6]. However, we propose some modifications and enhancements to improve modeling quality. Our approach is mainly divided into five steps: encoding genetic sequences by RAAA; selecting the most relevant sites; clustering the AAindex1 data set based on selected sites; encoding the selected sites by a representative from each cluster; and modeling antigenic variants by a classifier. The general schema of our pipeline is shown in Figure 1.

Data preparation

Our approach relies on three database types, each of which requires specific preparation in order to be used in the computational pipeline.

Simplified Amino Acid Alphabets. Apart from the standard amino acid alphabet with 20 letters, there are various RAAAs, in which the number of letters is less than 20. Typically, an RAAA is obtained by grouping the 20 amino acids. There are several strategies to perform this, some of which have been described [29]. For example, the set of 20 amino acids can be divided into three groups based on Van Der Waals volume by setting three ranges (0–2.78, 2.95–4.00, 4.43–8.08), resulting in three partitions: GASCTPD; NVEQIL; and MHKFRYW. This permits new interpretation of the mutation from a different point of view, such as change in hydrophobicity. In total, 40 published RAAAs were collected [8, 29, 38] and are presented in Table 1.

HI Assay Database. Typically, an HI assay database record includes three fields of information: test virus identifier; reference antiserum identifier; and HI titer. Sometimes additional metadata, such as experiment date, may be appended. HI assay results can be presented in four forms: raw HI titer; standardized HI titer; antigenic distance; and antigenic variant. At this point, we only used the antigenic variant obtained via the antigenic distance threshold. We employed Eq. (1) with threshold 4 for calculating the antigenic variant. Duplicated entries were averaged in terms of titer. Therefore, each test/reference virus combination is unique within the database. Here, we considered two subtypes in the influenza vaccine, H1N1 and H3N2. The HI assay database was taken

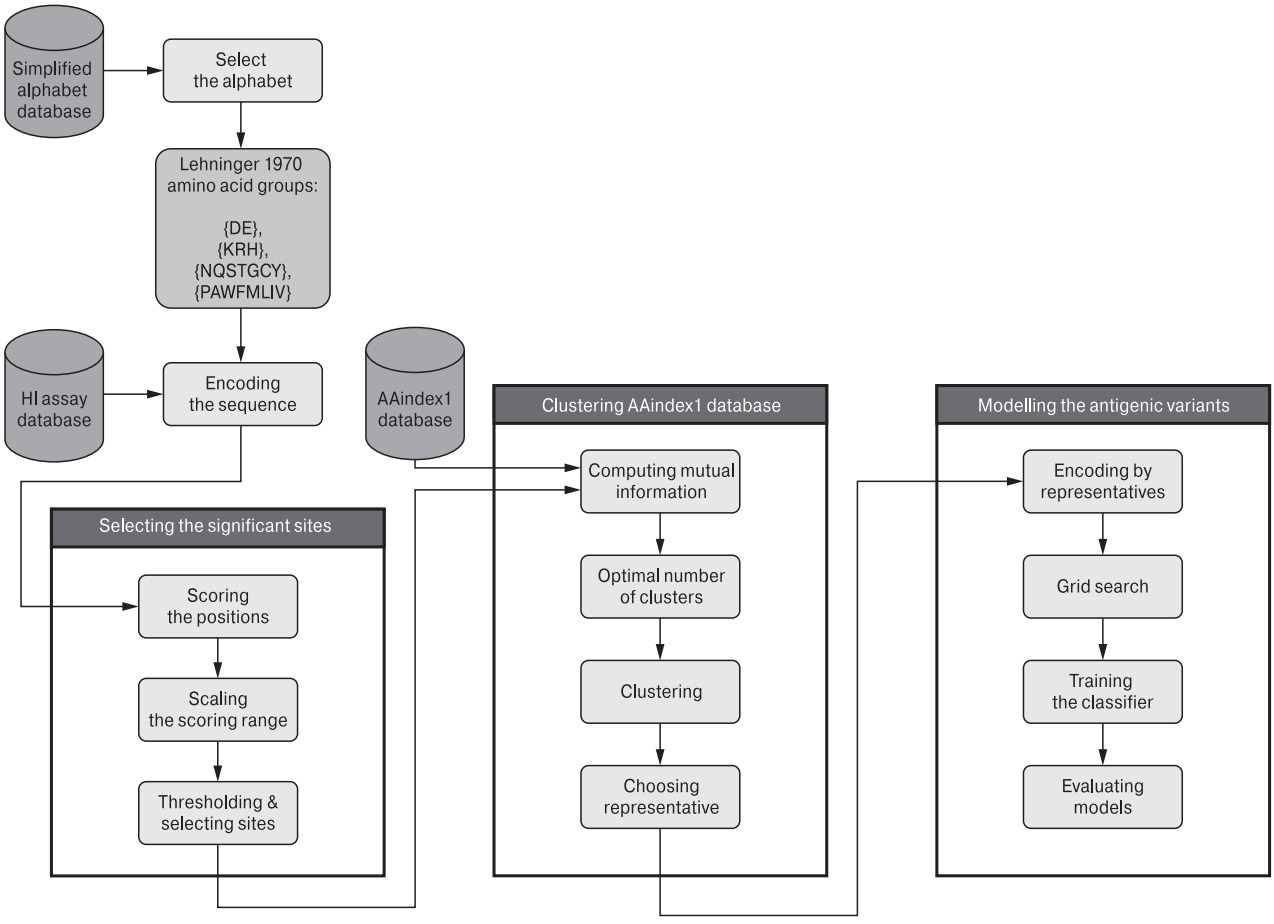

Figure 1. General scheme of the computational pipeline

Comments. Computational pipeline consists of five parts: encoding HA sequences by a reduced amino acid alphabet; selecting significant sites; clustering the AAindex1 database using mutual information of selected sites; encoding the sites by a representative from each cluster; and finally training the classifier.

from references [13, 34]. The final obtained database had 7449 H3N2 and 3747 H1N1 entries. There were 506 viruses for the H1N1 subtype (506 test against 44 references) and 772 for H3N2 (666 test against 130 references).

AAindex1 Database. The latest version of AAindex1, ver. 9.2, consists of 566 entries. A typical database entry includes a vector of 20 numbers, each of which is assigned to a standard amino acid. Since the range of numbers in vectors varies within the database, we individually scaled each vector into the unit interval *[0,1]*. After removing vectors with missing values, 553 remaining entries were used for analysis.

Encoding of HA sequences

Here, we use RAAAs to take into account the impact of the mutation on antigenic evolution from different physicochemical (amino acid) perspectives. The first step of encoding the sequence by RAAA is selecting an arbitrary amino acid from each group

Table 1. The list of alternative and standard amino acid alphabets employed to encode the protein sequences in our experiments

Name of alphabet	Groups	Method of obtaining the alphabet
Standard	A, C, D, E, Q, F, Y, G, H, I, V, K, R, L, M, N, P, S, T, W	
Hydrophobic	RKEDQN, GASTPHY, CVLIMFW	Amino acid physicochemical attributes
Van Der Waals Volume	GASCTPD, NVEQIL, MHKFRYW	
Polarity	LIFWCMVY, PATGS, HQRKNED	
Polarizability	GASDT, CPNVEQIL, KMHFRYW	
Mahler 1966	DE, KRH, QN, ST, P, CM, WYF, GALIV	
Lehninger 1970	DE, KRH, NQSTGCY, PAWFMLIV	
Dickerson 1983	DENKRQH, STGPACWY, FMLIV	
Taylor 1986	DE, N, KRH, Q, T, SGAC, P, YWF, M, LIV	
Weathers 2004	DENRH, KQST, GPACM, WYFLIV	
SE-B(14)	A, C, D, EQ, FY, G, H, IV, KR, LM, N, P, ST, W	Substitution frequency — Structural alignment
SE-B(8)	AST, C, DHN, EKQR, FWY, G, ILMV, P	
Risler 1988	D, E, N, KRQ, S, T, G, P, H, A, C W, YF, ML, IV	
Riddle 1997	DE, NKRQS, THA, GP, CWYFMLIV	
Mirny 1999	DE, KR, NQST, GP, HWYF, ACMLIV	
Prlic SDM12 2000	D, N, EKR, QST, G, P, H, A, C, W, YF, MLIV	
Prlic SDM17 2000	D, EK, N, R, Q, S, T, G, P, H, A, C, W, Y, F, M, LIV	
Melo 2005	DENKRQSTP, GA, H, C, WYFMLIV	
Robson 1976	DKR, EA, GP, STNQ, H, C, WY, FMLIV	Spatial frequency — Protein blocks
Solis(G) 2000	D, N, S, T, G, P, H, C, Y, EKRQAWFMLIV	
Solis(D) 2000	DNS, EKRQ, TH, GP, AM, C, W, F, YL, IV	
Rogov 2001	DNSTA, EKRQ, G, P, H, C, W, M, YFLIV	
Etchebest 2007	DN, EKRQ, SH, TC, G, P, WYF, AML, IV	
Solis GBMR4 2009	DENKRQSTA, G, P, HCWYFMLIV	
Zuo 2009	DN, E, KRQ, SH, T, G, P, A, C, WYF, M, L, IV	
Dayhoff 1978	DENQ, KRH, STGPA, C, WYF, MLIV	Substitution frequency — Sequence alignment
Murphy 2000	DENQ, KR, ST, G, P, H, A, C, WYF, MLIV	
Cannata 2002	D, E, N, KR, Q, ST, G, P, H, A, C, W, Y, F, ML, IV	
Fan 2003	DEQ, KR, STA, G, P, NH, C, WYF, ML, IV	
Li 2003	DE, KRQ, ST, G, P, NH, AC, WYF, ML, IV	
Edgar Se-B 2003	DN, EQ, KR, STA, G, P, HW, C, YF, MLIV	
Edgar Se-V 2003	DEN, KRQ, STA, G, P, H, C, W, YF, MLIV	
Kosiol 2003	DENKRQSTGPHA, C, W, YF, MLIV	
Anderson 2004	D, E, KRQ, NS, T, G, P, H, A, C, WYF, ML, IV	
Lenckowski 2007	DSHFM, ERQL, KPAC, NTWY, GIV	
Crippen 1990	ENRSGHY, DKQTPW, AV, CFMLI	Spatial frequency — Contact potential
Maiorov 1992	DENQ, KR, G, P, AV, STHWY, CFMLI	
Thomas 1996	DE, KR, QSTNGPH, C, AWYFMLIV	
Wang 1999	DE, NKRQS, GP, THA, CWYFMLIV	
Ceiplak 2001	DENRQSTG, K, HA, CWYMV, FLI	
Liu 2002	DE, KR, NQSTGPHY, ACW, FMLIV	

Note. The alphabets are borrowed from [8, 29, 38]. The classification of alphabets is borrowed from [29].

in the alphabet as a group representative. Further, we replace all members of the group with its representative in the protein sequence. This step does not influence data if the standard amino acid alphabet is chosen since this alphabet has 20 groups, not less.

Selection of high impact sites

The model's input is a feature vector produced from encoded relevant sites in the genetic sequence. The model utilizes these sites for reconstructing the relationship between genetic and antigenic evolution in the feature space. Therefore, it is necessary to measure the relevance of site mutations according to the antigenic variation. Cui et al. [6] proposed measurement by introducing the below score for the site's antigenic significance:

$$S_i = |\Phi_i| \times E_i \quad (3)$$

where i is the index of the site in the sequence, S_i is the significance score, and E_i is Shannon's entropy of site i in the whole database as computed by the following formula:

$$E_i = -\sum_{j=1}^{20} P_{i,j} log P_{i,j} \quad (4)$$

where $P_{i,j}$ is the probability of amino acid j occurrence at position i. Φ_i is a coefficient expressed with the following formula:

$$\Phi_i = \frac{(N_{11} \times N_{00} - N_{10} \times N_{01})}{\sqrt{N_{X1} \times N_{X0} \times N_{1Y} \times N_{0Y}}} \quad (5)$$

where N_{mn} *(m, n ∈ {0, 1})* is the number of HI entries with $X = m$ and $Y = n$. The variable X represents the occurrence of mutation at site i (0 or 1 for conserved or mutated cases, respectively). The variable Y expresses the antigenic relationship between the test-reference pair of viruses in HI entries. If the test and reference are antigenically similar, the Y variable value is zero. Otherwise, they are variants, and it takes the value of one. $N_{X,n}$ denotes the number of entries with $Y = n$, whereas X can take any value from {0,1}. Similarly, $N_{m,Y}$ represents the number of entries with $X = m$, while Y has a value from {0,1}. Note that all variables in Eq. (5) are calculated only for site i. In the case of a conserved site, the significance score is set to zero.

The application of Eq. (3) can be extended to sequences encoded by RAAAs. Encoding genetic sequences by such an alphabet notably changes the entropy and Φ values and, accordingly, the significance score. The significance score for all sites obtained by applying a RAAA is further scaled into the unit interval *[0,1]*. This allows us to compare the significance of a specific site considering different alphabets. The final high-impact sites are determined by setting a threshold on the results of the scaled significance score. The threshold value is selected from the set {0.2,0.3,0.4,0.5,0.6,0.7,0.8}. It's worth noting that a site is selected if its scaled significance score is higher than the target threshold. Obviously, decreasing the threshold leads to an increase in the number of selected (high-impact) sites.

Clustering the AAindex1 database

The AAindex1 database is used to perform the second stage of encoding. We select some entries from AAindex1 (called representatives) that are further used to encode the genetic information of obtained selected sites in the previous step. It is known that there is a high correlation between AAindex1 entries. Therefore, we cluster them and choose a representative from each cluster to diminish the number and correlation of final features. Clustering should be performed so that the objects of a cluster have almost the same encoding impact on antigenicity modeling.

Computing Mutual Information. To cluster the AAindex1 database, we create a feature vector for each entry by a similar scenario as described [6] with a modification for RAAAs. In the suggested method, the feature vector characterizes the AAindex1 entry by mutual information (MI). The MI value expresses not only the significance of genetic information but also the impact of encoding for a selected site individually. Note that the size of the feature vector for clustering AAindex1 is the same as the size of selected sites. Indeed, each element of the feature vector is the measure of mutual dependency between the changes in a selected site, encoded by an AAindex1 entry, and antigenic variants within the HI database.

The number of amino acids in the RAAA is less than in the standard alphabet. Thus, a question arises on how encoding is carried out using AAindex1 regarding a RAAA. In order to solve this issue, we define a new database, called pseudo-AAindex1, derived from the original AAindex1 database. The procedure of generating pseudo-AAindex1 is described in Figure 2.

As previously stated, each amino acid group has a representative, which replaces all amino acids of the group in protein sequences. In order to assign a value to the representative, we compute the average of AAindex1 values for the amino acids within the group. This allows each amino acid to participate and have its own effect through the representative. Thus, a pseudo-AAindex1 is created for each RAAA, making it possible to calculate the mutual information in RAAA encoding. For simplicity, we hereafter refer to both the original AAindex1 and the pseudo-AAindex1 simply as AAindex1.

Determining the of optimal number of cluster. When considering an alphabet, we create a feature vector for each AAindex1 entry, the size of which depends on the number of determined high-impact sites. Before clustering AAindex1, it is necessary to determine the optimal number of clusters. Indeed, this

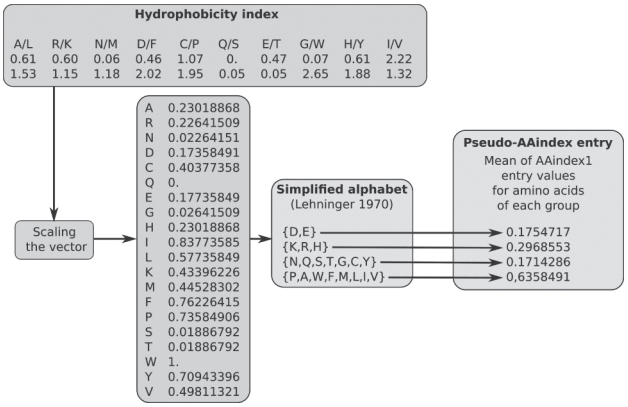

Figure 2. Generation of the pseudo-AAindex1 database from the hydrophobicity index
Note. The pseudo database is created based on the selected RAAA. Note that the value assigned to each group is the average of the group's amino acid values in the scaled AAindex1 vector.

number affects the final feature vector, which is used for antigenic variant modeling. For this purpose, we conduct a comprehensive search for the optimal number by employing three algorithms: K-means; agglomerative clustering with different linkage criteria; and spectral clustering.

First, we determine the number of unique feature vectors. Clustering is not required if the number is less than a threshold (e.g., five). When the number of unique vectors is more than the threshold, we cluster the set of vectors, while the number of clusters starts from two and increases up to ten.

Generally, six clustering variants are applied, including: K-means; agglomerative clustering with four different criteria (ward, average, complete/maximum, and single/minimum); and spectral clustering. The obtained clustering from each algorithm is individually evaluated by four scores, including Silhouette, Calinski-Harabasz, Davies-Bouldin, and the sum of squared distances of objects to their closest cluster. Further, the results are plotted and manually checked to decide the optimal number of clusters for AAindex1 associated with an alphabet.

Clustering. Generally speaking, the aim of clustering is to decrease correlation between AAindex1 entries. This also leads to diminishing the number of features, which are used in the final classification. To cluster the AAindex1 database, we apply the associated clustering algorithm by which the optimal number of clusters was determined from the previous subsection. Next, we select a representative from each cluster. The representative of a cluster is the closest object to its center. The representative is further employed to encode the information of high-impact sites for the classification.

Classification of antigenic variants

We use the obtained cluster representatives to apply the secondary encoding. This is carried out by replacing an amino acid group representative in the selected sites with its numerical value from the cluster's representative. Then, we individually calculate the differences between the test and the reference strains for each HI assay database entry by subtracting their encoded selected sites (or feature vectors). If we denote the number of high-impact sites and number of clusters representatives with N and M, respectively, then the final feature vector has the size of $N \times M$.

Before performing the final classification, the last step is to determine the best classifier. To decrease the effect of the classifier on the results, we consider five different classifiers, including random forest, multilayer perceptron, logistic regression, support vector, and Gaussian naïve Bayes. Each classifier has its own parameters optimized through grid search (parameter list in Table 2).

Grid search is carried out by cross-validation with different parameter combinations. Note that the Gaussian naïve Bayes classifier has no parameters for grid search, but it assumes that features are independent. Thus, we perform principal component analysis on the feature matrix to decrease the dependency. A threshold on the percentage of variance explained by the selected components was set as a parameter for Gaussian naïve Bayes.

By comparing grid search results, we were able to choose the best classifier with high performance in terms of accuracy. Note that the selection of optimal classifier depends on the results of three procedures:

– Encoding by the alphabet (primary encoding);
– Selection of high-impact sites;
– Clustering the AAindex1 database and choosing representatives for secondary encoding.

Among these procedures, the first has the most decisive influence on classification results. In fact,

Table 2. Parameters used in the grid search

Method	Parameters	Total cases in gird search
Random forest	Criterion, n_estimator, min_samples_split	Fitting 5-fold cross-validation for each of 40 candidates, totaling 200 fits
Logistic regression	Solver, penalty, max_iter	Fitting 5 folds for each of 66 candidates, total 330 fits
Multilayer perceptron	Solver, learning_rate, activation, max_iter, learning_rate_init, hidden_layer_sizes	Fitting 5 folds for each of 648 candidates, total 3240 fits
SVM	Kernel, gamma, C, degree	Fitting 5 folds for each of 24 candidates, total 120 fits
Gaussian naive bayes	PCA_threshold	Fitting 5 fold for each of 3 candidates, total 15 fits

Note. Parameter names are based on the machine learning package Scikit-learn [22].

Table 3. The maximum accuracy was obtained by 10-fold cross-validation using different thresholds for scaled significance score

Subtype \ Threshold	0.2	0.3	0.4	0.5	0.6	0.7	0.8
H1N1	0.88	0.88	0.87	0.84	0.77	0.77	0.77
H3N2	0.92	0.92	0.92	0.9	0.88	0.85	0.83

Note. Increasing the threshold may lead to shorter feature vector length and consequent reduced accuracy.

it changes the amino acid space globally, resulting in different representations of genetic variation, as well as different relationships between genotype and phenotype.

Results and discussion

Considering all parameters, we ran 224 147 fits (41 alphabets × 7 thresholds × 781 5-fold cross-validations) for each subtype (H3N2 and H1N1) in the experimental data to obtain the best classifiers. Knowing the best classifier for each triple-combination case (subtype, alphabet, threshold), we performed a 10-fold cross-validation by applying its best classifier. A comprehensive report of the results, including the evaluation criteria, is publicly available at: github.com/viroinformatics/Simplified_Alphabets.

The maximum accuracy achieved by each threshold is presented in Table 3. Since the length of the feature vector is decreased by increasing the threshold, this also leads to accuracy reduction. From Table 3, it is observed that threshold 0.4 seems to be a good choice for modeling the antigenic variants. Compared with previous studies [10, 11], our results indicate a high degree of accuracy, especially for H3N2, which suggests potential application in the field of public health.

As expected, some RAAAs achieved the same accuracy as the standard amino acid alphabet. Table 4 presents the alphabets with the highest performance for different thresholds and subtypes. In the case of subtype H1N1 with thresholds 0.3 and 0.5, there are alphabets, the accuracy of which are slightly less (about 0.01) than the standard and Prlic-SDM12–2000 alphabets, but are not added to the table. Since prediction accuracy significantly drops from threshold 0.5 to threshold 0.8 Table 3), we did not consider their results in Table 4. Interestingly, the Risler-88 and Li-2003 alphabets are observed in the list of each subtype.

Moreover, the Cannata-2002 alphabet seems to be more informative for subtype H3N2 rather than for H1N1. In some cases, e.g., subtype H1N1 with threshold 0.4 and subtype H3N2 with threshold 0.3, the feature vector obtained from RAAAs is shorter in length than that obtained from the standard alphabet, while their accuracy is the same. This indicates that the amino acid space represented by the standard alphabet has redundant dimensions to express genetic variation of antigenic variants. Next, we briefly discuss each of the alphabets from Table 4.

Stephenson & Freeland analyzed 34 different RAAAs [29] and classified them into five classes based on how grouping was carried out. The classes are chemistry, sequence alignment, structural alignment, contact potential, and protein blocks. Of the alphabets in Table 4, four are based on sequence alignment methods, whereas two rely on structural alignment. Only one alphabet (Zou-2009) was created by protein blocks. The complete classification of RAAAs presented in Table 1 is based on published work [29].

Similarity between amino acids can be defined from various viewpoints, e.g., hydrophobic residues (I, V) and aromatic residues (F, W, Y). The main idea of constructing a RAAA is to use amino acid properties to define similarity, with placing of similar amino acids in a group. For example, the RAAA presented by Li et al. [20] was obtained from amino acids substitutions by scoring similarities that may be beneficial in recognition of protein folds. Their results imply that at least ten amino acid types are required to characterize protein complexity.

Cannata et al. [5] presented a method to produce RAAAs by scoring different amino acid compositions using a branch and bound algorithm and substitution matrix. Their alphabet belongs to the 'alignment-based methods' class of sequences. Furthermore, Lenckowski et al. [19] suggested an alphabet generated using a genetic algorithm and strategy based on global sequence alignment. Their results indicate that the proposed alphabet outperformed the standard amino acid set and other RAAAs in the sequence classification task. Andersen and Brunak's RAAA [1] includes 13 letters; it is also constructed based on se-

Table 4. High performance alphabets in our experiments by virus type and site selection threshold

Threshold	H1N1	H3N2
0.2	Standard, Risler-88, Li-2003, Anderson-2004	Standard, Risler-88, Cannata-2002, Zuo-2009
0.3	Standard*	Standard, Cannata-2002
0.4	Standard, Lenckowski-2007	Standard, Cannata-2002
0.5	Prlic-SDM12–2000*	Risler-88, Li-2003, Zuo-2009

Note. The amino acid groups for each alphabet are presented in Table 1. *There are other alphabets, not listed, whose accuracy was slightly less than the alphabets shown in the table.

quence alignment. In contrast, RAAAs proposed by Prlic et al. [23] and Risler et al. [25] are both derived by substitution frequency of structural alignments.

Zou et al. [42] applied reduced amino acid alphabets to predict defensin family and subfamily. They clustered amino acids by the protein blocks (PBs) method [7, 9], in which the distribution of amino acids in PBs was used to generate clusters of equivalent amino acids with respect to local structure. Indeed, this kind of alphabet can be considered a structural alphabet. Their results indicate that use of such alphabets can improve prediction accuracy with defensin family and subfamily. Surprisingly, no alphabet based on attributes of individual amino acids attained a high level of performance. Taken together, the high-performing RAAAs emphasize the role of structural features in antigenic evolution modeling.

By checking the high-impact sites in the Influenza Research Database (IRD) [40], we found that not only antigenic sites have a significant influence on antigenicity, but also other amino acids located in close proximity. The results indicate that all selected non-antigenic sites are related to immune responses. According to IRD, these have been experimentally determined to be T-cell epitopes, B-cell epitopes, and MHC-binding epitopes of different classes. This highlighted a caveat: In modeling of antigenic evolution, the model should consider not only the genetic information of antigenic sites, but also that of neighboring positions, as they may indirectly impact antigenicity. Note that feature vector construction relies on high-impact sites, but the evolutionary history showed that even one amino acid substitution can change the antigenic cluster of the influenza virus [28]. Such a substitution may present a low impact through the mutual information score. We believe a desirable model must take into account the effects of both high and low impact sites. The visualizations of selected high-impact sites for H1N1 (threshold 0.3), and H3N2 (threshold 0.4), are presented in Figure 3 (see cover III). These are cases with high accuracy and shorter feature vector length.

Various AAindex1 entries were designated as representatives during all experiments with optimized classifiers. The top ten entries and their frequencies are listed in Table 5. The complete list of AAindex1 entries and their frequencies is available (github.com/viroinformatics/Simplified_Alphabets).

It can be seen that the majority of AAindex1 attributes used in model construction are associated with charge properties. This emphasizes that antigenicity notably depends on protein conformation, which cannot be fully reflected in a one-dimensional representation of protein as a sequence. However, the model can capture some attributes information by encoding the genetic sequence using physicochemical properties presented in the AAindex1 database.

Table 5 also indicates that nine out of the ten most frequently AAindex1 entries are common in both subtypes. The last AAindex1 entry in the list of each subtype is different. To better understand the characteristics of entries in Table 5, we computed the Pearson correlation coefficient and visualized it in Figure 4 (see cover III). It is observed that

Table 5. List of the top ten most frequent AAindex1 entries in the experiments with optimized classifiers

ID	Description	Freq.
H1N1		
ANDN920101	alpha-CH chemical shifts (Andersen et al., 1992)	147
CHAM830104	The number of atoms in the side chain labelled 2+1 (Charton-Charton, 1983)	106
KLEP840101	Net charge (Klein et al., 1984)	97
CHAM830103	The number of atoms in the side chain labelled 1+1 (Charton-Charton, 1983)	92
FAUJ880111	Positive charge (Fauchere et al., 1988)	83
CHAM830107	A parameter of charge transfer capability (Charton-Charton, 1983)	83
VENT840101	Bitterness (Venanzi, 1984)	74
FAUJ880112	Negative charge (Fauchere et al., 1988)	70
FAUJ880105	STERIMOL minimum width of the side chain (Fauchere et al., 1988)	47
CHAM830105	The number of atoms in the side chain labelled 3+1 (Charton-Charton, 1983)	38
H3N2		
VENT840101	Bitterness (Venanzi, 1984)	119
CHAM830103	The number of atoms in the side chain labelled 1+1 (Charton-Charton, 1983)	117
FAUJ880111	Positive charge (Fauchere et al., 1988)	101
ANDN920101	alpha-CH chemical shifts (Andersen et al., 1992)	101
KLEP840101	Net charge (Klein et al., 1984)	88
FAUJ880112	Negative charge (Fauchere et al., 1988)	87
CHAM830107	A parameter of charge transfer capability (Charton-Charton, 1983)	66
CHAM830104	The number of atoms in the side chain labelled 2+1 (Charton-Charton, 1983)	60
FAUJ880105	STERIMOL minimum width of the side chain (Fauchere et al., 1988)	59
FAUJ880109	Number of hydrogen bond donors (Fauchere et al., 1988)	58

the majority of entries in Table 5 are not correlated, with two exceptions: FAUJ880111/KLEP840101 and FAUJ880105/CHAM830103.

Although the uncommon entries between H1N1 and H3N2 are different, it can be seen that they are correlated. In addition to the correlation matrix, we used principal component analysis (PCA) to identify the main components of 11 distinct AAindex1 entries in Table 5 and their expression in terms of explained variance. Figure 5 indicates that the first six components describe more than 90% of the explained variance. Seven and nine components represent 95% and 99% of the explained variance, respectively.

We also considered the performance of classifiers for antigenic variant modeling. Among five classifiers, random forest and multilayer perceptron outperformed others, in terms of accuracy, for both the H1N1 and H3N2 subtypes. The Gaussian naive Bayes classifier gave the worst results, so it may not be suitable for this kind of modeling.

In summary, the outstanding ability of our approach is based on redefining the mutation by RAAA and amino acid attributes used for encoding through a two-fold procedure. The primary encoding plays the main role with high priority, whereas secondary encoding has a supplementary role. From one point of view, the primary encoding determines the high-impact sites, while the secondary encoding gives the numerical interpretation to the genetic information of selected sites. From another point of view, the primary encoding interprets the mutation, and the secondary encoding reconstructs the specific relationship between genetic and antigenic differences (for the test and reference strains).

The proposed two-fold encoding approach revealed some aspects of mutations related to the antigenicity. Our findings indicate that encoding associated with structural or charge properties of the protein dramatically impacts the performance of the antigenic model. This is in agreement with recent studies done by other researchers [14, 39]. In addition, RAAA encoding can lead to a smaller feature space dimension, while performance is maintained or improved. So far, this approach was applied only to seasonal human influenza strains. However, there are no theoretical limitations that would prevent further testing as a universal computational model for predicting antigenicity in other influenza subtypes, such as zoonotic H5, H7, H9, or other relevant influenza A subtypes that cause sporadic human infection.

Conclusion

Determining the degree of antigenic similarity between influenza virus strains is crucial in choosing candidate vaccine strains and subsequent timely vaccine production. Currently, the degree is measured via HI assay, a widespread laboratory procedure. Although HI assay is the gold standard method,

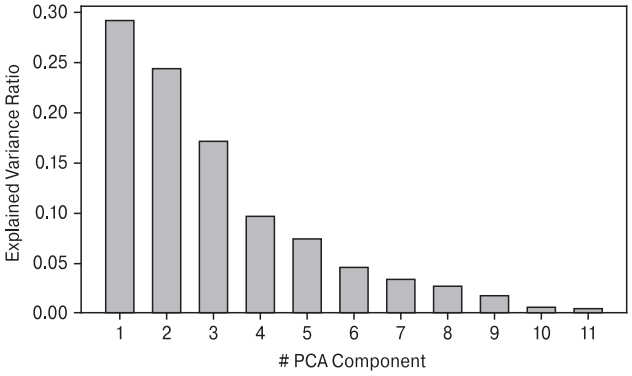

Figure 5. Explained variance ratios for PCA analysis components

Note. Analysis was performed for 11 unique AAindex1 indices from Table 5. The result shows that the first six components represent more than 90% of the explained variance.

it suffers from several shortcomings. Therefore, it has been suggested to employ computer-aided models as auxiliary tools to assess preliminary information about viral antigenicity prior to HI assay.

A notable problem in modeling antigenic evolution is the representation of genetic information to better express the relationship between genetic and antigenic variations. This paper proposes a two-fold encoding approach to genetic information using both a reduced amino acid alphabet (RAAA) and an amino acid index database. By applying a RAAA, we redefine the mutation as changes between amino acid groups of the alphabet, while the output sequence of the primary encoding is still alphabetical. The secondary encoding uses representatives from the AAindex1 database to convert the alphabetical sequence of the primary encoding into the numerical. The experimental results indicate that models built using RAAA encoding are able to achieve the same accuracy as models using the standard amino acid alphabet. The RAAA-based approach, however, features reduced computational complexity and associated cost.

Moreover, the suggested encoding can reveal the amino acid attributes which drive antigenic evolution. In agreement with previous studies, we find that structural and charge characteristics are the most beneficial in modeling antigenic evolution. Although the results obtained by our approach are desirable and promising, they are achieved by taking into account only high-impact sites. It is known that even one substitution can change the antigenic cluster, so we believe that further incorporating the role of low-impact sites into the model may enhance its accuracy and prediction potential; this will be addressed in future studies. Additionally, the model can be improved by: introducing new reduced amino acid alphabets; employing more significant and descriptive criteria for selecting key sites; and incorporating neighboring amino acid effects into the model.

Computational approaches for predicting antigenic properties from genetic sequence are also quite relevant for highly virulent influenza viruses. Laboratory testing of these pathogens requires high biosafety certification levels, and such analysis is not only time-consuming and labor-intensive, but also costly. Unlike current laboratory approaches, computational prediction of antigenic properties from viral sequence has the potential to enable rapid, large-scale antigenic characterization of influenza viruses. It is worth mentioning that application of our approach is not limited to modeling of antigenic evolution. It can be used in modeling any phenotype that is based on protein sequence, such as interactions with monoclonal antibodies.

Acknowledgments

Our work was performed using the "Uran" supercomputer (IMM UB RAS). The authors would like to thank Edward S. Ramsay for his valuable improvements on the manuscript.

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Поступила в редакцию 31.05.2022
Принята к печати 06.08.2022

Received 31.05.2022
Accepted 06.08.2022

Иллюстрации к статье «Кодирование с помощью сокращенного аминокислотного алфавита и его влияние на моделирование антигенной эволюции гриппа» (авторы: М. Форгани, А.Л. Фирстков, М.М. Аляннеджади, Д.М. Даниленко, А.Б. Комиссаров) (с. 837–849)

Illustrations for the article “Reduced amino acid alphabet-based encoding and its impact on modeling influenza antigenic evolution” (authors: Forghani M., Firstkov A.L., Alyannezhadi M.M., Danilenko D.M., Komissarov A.B.) (pp. 837–849)

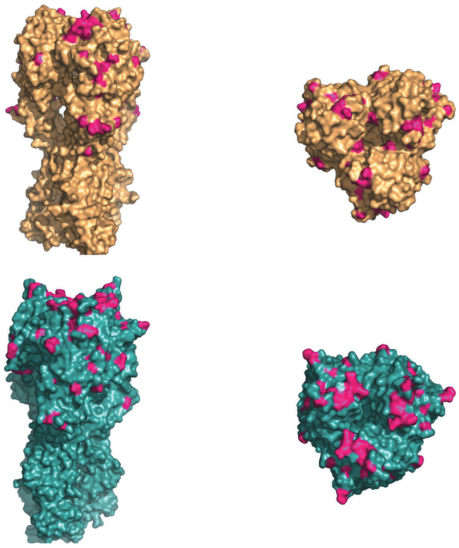

Figure 3. Visualization of high-impact sites on the surface of hemagglutinin protein by PyMOL [26]
Note. Top — H1 protein (PDB ID: 1RUY [3, 12]). Bottom — H3 protein (PDB ID: 5THF [3, 33]). Note that the highlighted sites include not only the antigenic sites but also those experimentally determined as T-cell epitopes, B-cell epitopes, as well as MHC-binding epitopes of different classes.

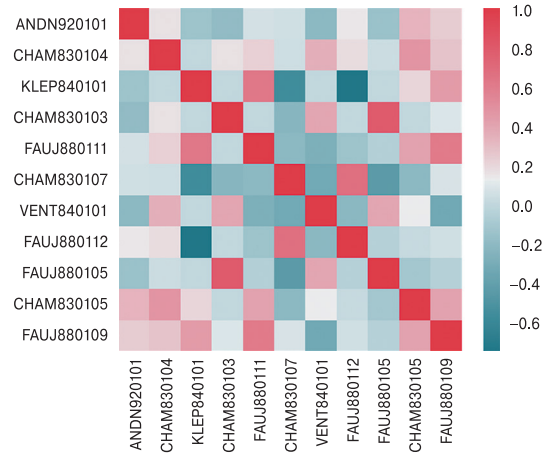

Figure 4. Correlation matrix of 11 unique AAindex1 entries from Table 5
Note. Majority of indices have low correlation.