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Research article

Designing an mRNA-based universal vaccine for influenza A virus using an *in silico* approach

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ABSTRACT

Introduction: The influenza virus undergoes constant mutation, necessitating annual updates to the seasonal flu vaccine. Traditional vaccine methods struggle to keep pace with the rapid mutation rate of this virus. This study aimed to develop a universal mRNA vaccine using immunoinformatic approaches to effectively target various strains of influenza A viruses (IAVs). Specifically, this study identified epitopes capable of eliciting T-cell-mediated immune responses for inclusion in vaccine design. **Materials and Methods**: Immunoinformatic tools from the Immune Epitope Database (IEDB) were used to direct neutralizing antibodies (nAbs) against hemagglutinin (HA) molecules. T lymphocyte epitopes were predicted using multiple sequence alignment from Clustal Omega. These epitopes were filtered through a pipeline to assess their affinity for major histocompatibility complex (MHC) class 1 molecules and their conservation across various IAV strains. **Results:** After data cleaning, 82,200 of the 91,093 HA amino acid sequences of 15 human-infected IAV strains were eligible for subsequent epitope prediction and conservation assessment. The lengths of the HA amino acid sequences across the 15 strains predominantly fell within the range of 560–567 amino acids. MHC class 1 interaction analysis revealed 16 antigenic sequences generally located in the stalk domain of HA. Among those, 11 were found to have a consensus. Five consensus sequences with strong binding scores were converted into universal mRNA sequences that were ready to be used for subsequent vaccine research. **Conclusion:** Consesus and antigenic HA sequences were successfully designed for use against diverse IAV strains, particularly those circulating in Vietnam. The use of immune-informatics tools with stalk-directed strategies has been shown to be an effective step in viral vaccine development.

Key words: Epitopes, immune-formatics, in silico design, influenza A virus, mRNA vaccine

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INTRODUCTION

New strains of the influenza A virus (IAV) emerge every 2–5 years, lasting for 3–6 months and varying based on the country and region[1]. Terming the "seasonal flu" due to its link with climate and weather[2], a typical influenza virus particle comprises approximately 500 hemagglutinin (HA) molecules and 100 neuraminidase (NA) molecules, both of which are pivotal in the virus's ability to cause influenza[3]. IAV is associated with 16 forms of hemagglutinin (H1 to H16) and 11 forms of neuraminidase (N1 to N11), which are viral antigenic proteins used to distinguish subtypes[4].

New mutations within HA and NA membrane glycoproteins enable the virus to evade the host immune system. Neither natural infection nor vaccination can confer permanent protection against virus strains[3,4]. The extensive viral diversity poses challenges for vaccine development, resulting in low immunogenicity indices and making it difficult to meet annual immune needs[5]. Current seasonal influenza vaccines are updated annually, and global surveillance is needed to predict future circulating strains. Southeast Asia and sub-Saharan Africa exhibit the highest mortality rates attributed to IAV infections. The annual flu epidemic is projected to cause 3–5 million severe cases and 290,000–650,000 respiratory deaths globally[6]. For seasonal flu, most people recover within a week without medical intervention however, high-risk groups, including those aged 75 and older, children under 5, and individuals with underlying illnesses, can suffer severe illness and mortality[7]. In the past two decades, Vietnam has experienced annual circulation of H5N1, a highly pathogenic avian influenza (HPAI) virus, leading to substantial economic losses and a notable fatality rate[8]. From 2017 to 2022, scientists identified the H5N1 virus strain in live bird markets across various provinces, which poses a significant risk for avian influenza transmission to humans[9]. During 2009–2010, H1N1 was transmitted from swine to humans, resulting in an outbreak in Vietnam[10].

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The success of mRNA vaccines in combating COVID-19 [11] has made them promising candidates for the development of innovative universal influenza A virus (IAV) vaccines. Not only do they demonstrate comparable potency to traditional methods, but they also significantly expedite vaccine development and distribution. Although this approach has proven effective for single-antigen vaccines such as COVID-19, there is room for improvement in tackling more complex multiantigen vaccines. In terms of translation, mRNA immunizations outshine DNA vaccinations as they circumvent the need for nuclear processes [12]. The ability of this new technology to modify formulas and alter nucleotide sequences is a major advantage over conventional methods, enabling the formulation of defenses against future mutant strains. Moreover, these vaccines can be manufactured in the same facility using a uniform method, leading to notable time and cost savings as well as increased flexibility [11,13]. The focus on neutralizing antibodies (nAbs) revolves around their precise targeting of the HA protein, which effectively prevents influenza virus infection [4]. Therefore, this study aimed to construct a universal mRNA vaccine tailored to the HA of IAV, particularly endemic strains circulating in Vietnam.

MATERIALS AND METHODS

Workflow of the study

This study was conducted in five stages: 1. Acquisition of HA amino acid sequences from human-infected IAV strains. The human-infected strains were identified by using the "WHO Influenza Virus Traceability Mechanism (IVTM)" (https://extranet.who.int/ivtm2/), and the sequences were obtained from the National Center for Biotechnology Information (NCBI) protein database (https://www.ncbi.nlm.nih.gov/protein); 2. The application of noise elimination techniques to identify eligible sequences; 3. Prediction of T-cell epitopes through major histocompatibility complex (MHC) class I binding analysis; 4. Multiple Sequence Alignment of the HA Amino Acid Sequences; 5. Transformation of antigenic conserved regions (CRs) into mRNA sequences. [Figure 1]

Acquisition of hemagglutinin amino acid sequences from human-infected IAV strains

The IVTM database served as the source for retrieving influenza type A strains infecting humans. The amino acid sequences of the HA glycoprotein were extracted from the NCBI protein database using a specific syntax and keyword: *"hemagglutinin [All Fields] AND "influenza A virus [porgn] AND viruses [filter] AND "subtype [All Fields] NOT "partial [All Fields] NOT "fragment [All Fields] NOT "mixed [All Fields] AND "aa [SLEN]: "aa [SLEN]*. The term "Subtype" was replaced with the respective strain name, and "aa" was replaced with the designated sequence intervals. To determine the maximum length of HA amino acid sequences for each strain, a preliminary investigation was conducted, establishing the following sequence intervals: 1-100, 101-200, 201-300, 301-400, 401-500, 501-559, 560-566, 567-700, and 701-760. The interval with the highest number of reported sequences was chosen as the representative sequence interval for each strain, and subsequently, the GenPept (database of GenBank gene products) files of these sequences were downloaded.

Application of noise elimination techniques to identify eligible sequences

Data that contain a large amount of unnecessary information are called "noisy data", which in this case were sequences from unwanted strains. To perform the data cleaning, two Python scripts were used. At the first cleaning stage, by looking at the information in the GenPept files of the HA amino acid sequences, a sequence from the desired strain and its accession number were determined. The FASTA sequences were downloaded from the results. After the first cleaning stage, SeqKit, a toolkit for FASTA/Q file manipulation, was used to convert the FASTA files from multiline format into single-line format, allowing us to run the second script. The second script was used to check the quality of the FASTA files. If any sequences that contained X, an unknown amino acid, were considered to be of low quality, they were excluded from the final database.

Prediction of T-cell epitopes through MHC class I binding analysis

The binding between T-cell epitopes and their respective MHC class I molecules was simulated. Human MHC, also known as human leukocyte antigen (HLA) class I, plays a pivotal role in the adaptive immune system by presenting cytosolic peptides, including viral peptides, on the cell surface for T-cell recognition [14]. The most frequently occurring HLA alleles among the Kinh Vietnamese population were selected, including A*11:01:01, B*15:02:01, and C*07:02:01, for analysis [15]. With each strain, one sequence was randomly chosen as a representative, and the T-Cell Epitopes - MHC Binding Prediction tool from the IEDB Analysis Resource was used to predict the subsequent ability to bind to a specific MHC class I molecule. Subsequently, only the top 5 antigenic regions with the

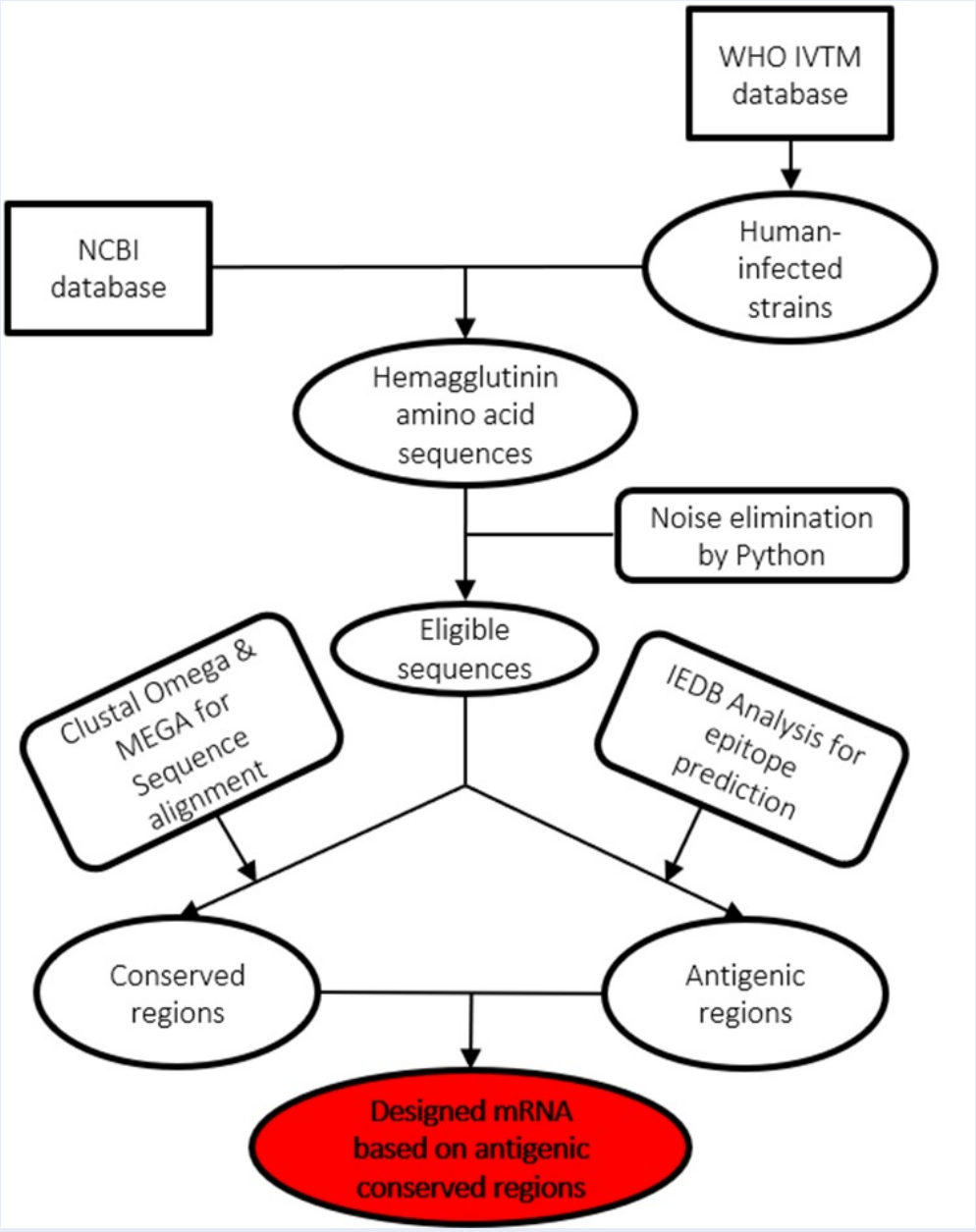

Figure 1: Workflow of the study. The human-infected strains identified by using the "WHO Influenza Virus Traceability Mechanism (IVTM)" were used as criteria to extract HA amino acid sequences from National Center for Biotechnology Information (NCBI) protein database. Then, Python was applied to remove noise for identification of the eligible sequences. The antigenic regions were predicted through major histocompatibility complex (MHC) class I binding analysis and the conserved sequences were obtained via Multiple Sequence Alignment of the antigenic HA amino acid sequences. Finally, the antigenic conserved regions (CRs) were converted into mRNA sequences.

highest binding scores for each strain were retained for further analysis of each HLA allele.

Multiple Sequence Alignment of the Hemagglutinin Amino Acid Sequences

The selected antigenic regions were assessed for their conservation among different strains. The after-cleaning sequence database was used, and the seeded guide tree and HMM profile profile techniques from the Clustal Omega server [13] were employed to generate sequence alignments. The parameters were set to default, but the output format was changed to Pearson/FASTA since the results were visualized using MEGA software. Due to the diversity among different strains, any antigenic regions that were above the threshold of 80% consensus were regarded as conserved.

Transformation of antigenic conserved regions into mRNA sequences.

Using the International Union of Pure and Applied Chemistry (IUPAC) code [16,17], the amino acid sequences of the conserved antigenic regions (CRs) were converted into mRNA sequences (Table 1).

Additionally, these CRs were combined with the representative HA amino acid sequences from 15 strains to construct a maximum-likelihood phylogenetic tree using the phylogenetic tool from MEGA Software. The tree was used to observe the diversity and distribution of HA-subtype sequences and the coverage of CRs within these strains.

RESULTS

Extraction and noise elimination of hemagglutinin amino acid sequences from IAV human-infected strains

Based on the IVTM database, 13 strains were identified as causative agents of endemic influenza, namely, H1N1, H1N2, H2N2, H3N2, H5N1, H5N6, H6N, H7N2, H7N3, H7N7, H7N9, H9N2, and H10N8 [18]. Additionally, H10N3 and H10N7 were confirmed to be human-infected strains [19,20], although they were not included in the IVTM database. In total, 15 strains were implicated in human infections. Presently, H1N1 and H3N2 stand out as the most common subtypes of IAV responsible for seasonal flu [21,22]. These two strains had the greatest number of reported HA amino acid sequences (30,866 for H1N1 and 41,009 for H3N2) among the 15 strains (Table 2). The lengths of the HA amino acid sequences across the 15 strains predominantly fell within the range of 560–567 amino acids.

From 91,093 HA amino acid sequences of 15 human-infected IAV strains, 82,200 were obtained after data cleaning and were eligible for subsequent epitope prediction and conservation assessment.

Prediction of T-cell epitopes through MHC class I binding and extraction of conserved sequences using

With the T-cellcell epitope-MHC binding prediction tool from the IEDB Analysis Resource, 16 epitopes were selected (Table 3). Many strains had the same epitopes. The antigenic regions with the highest binding scores were HLA-A*11:01:01, followed by HLA-B*15:01 and HLA-C*07:02.

After multiple sequence alignment, 11 out of 16 of the selected epitopes were shown to be conserved, and the five most antigenic epitopes (STIDGITNK, KAIDGVTNK, KTNEHQIEK, SAIDQITGK, and AAIDQITGK) (Table 3) were selected for mRNA universal vaccine design.

The mRNA sequences encoding

The antigenic conserved regions (CRs) mostly ranged from 380-400 amino acids (Table 4), and based on the NCBI database, these regions are located in the stalk domain of HA (HA2), which frequently ranges from 345-566 amino acids. Finally, the IUPAC codes were used to convert the amino acid sequences of the genes into primary and secondary consensus mRNA sequences (Table 4).

The representative HA amino acid sequences from 15 strains and the 5 obtained CRs, CR1-5, were used to construct a phylogenetic tree (Figure 2).

The tree provides a comprehensive overview of the phylogenetic diversity and distribution of IAV strains based on HA sequences. The results revealed that the 15 HA subtypes formed distinct groups. The first group comprised H1N1, H1N2, H2N2, H5N1, H5N6, and H6N1, while the second group included H3N2, H7N2, H7N3, H7N7, H7N9, H9N2, H10N3, H10N7, and H10N8.

DISCUSSION

The diversity and distribution of IAV strains analyzed using their protein sequences were previously investigated [23,24]. The results, particularly regarding the HA subtypes, revealed a strong resemblance to the diversity and distribution patterns observed in our study. Notably, the tree analysis illustrated that each CR effectively encapsulated its corresponding strains. Additionally, CR3, which includes H2N2 and H3N2, exhibited coverage extending to the HA of influenza

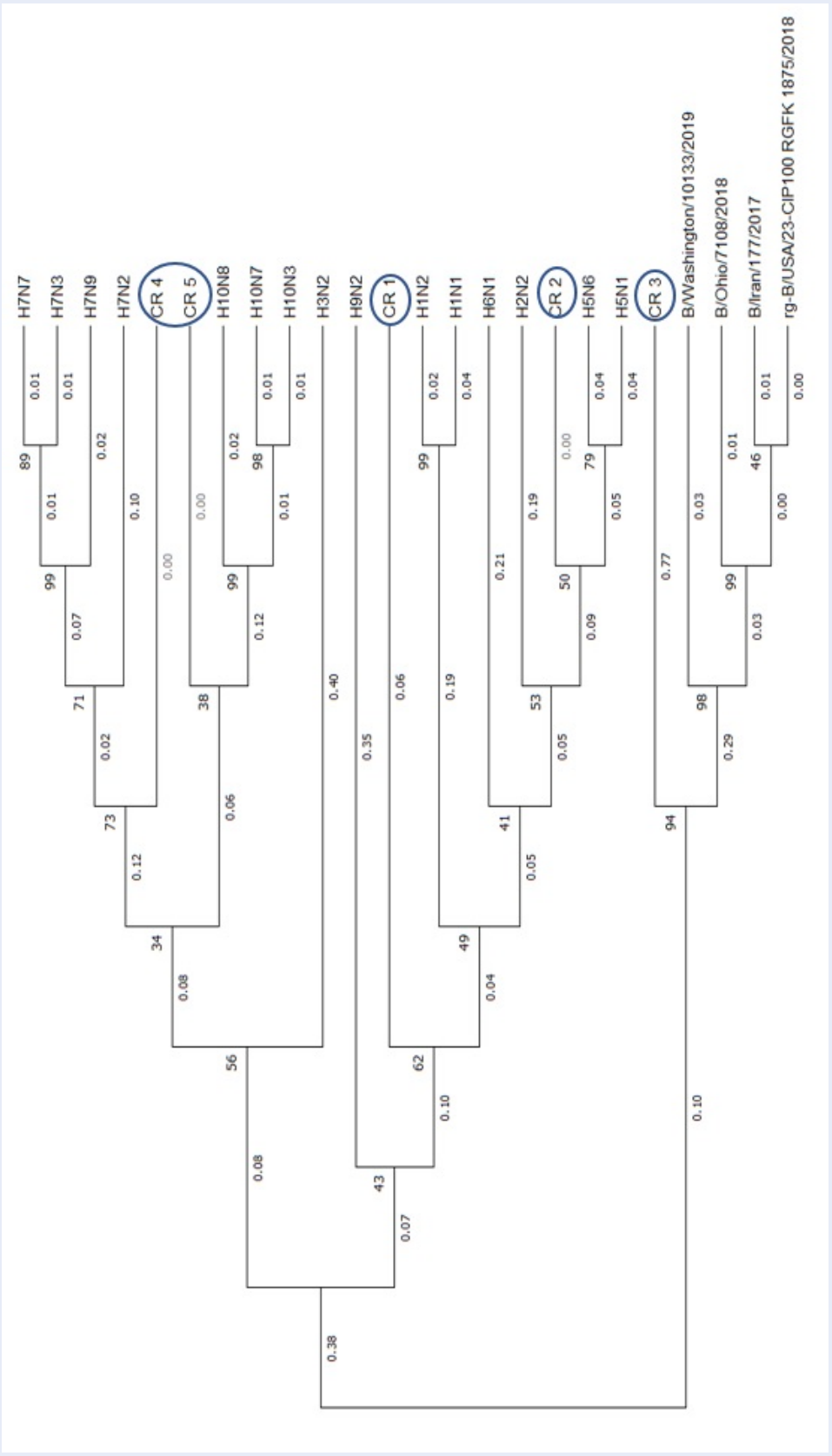

Figure 2: Phylogenetic relationship of the influenza A and influenza B hemagglutinin proteins and the variant of antigenic conserved regions (CRs) between type A strains. The representative strains for each HA subtype were aligned using Clustal Omega alignment, and a neighbor-joining phylogenetic tree was constructed using MEGA software.

Table 1: IUPAC codes for amino acids and ribonucleotides [17]

Amino acid		Ribonucleotide	
S	Serine	Y	Pyrimidine - U or C
K	Lysine	R	Purine - A or G
A	Alanine	W	2 Hydrogen bonds
T	Threonine	S	3 Hydrogen bonds
I	Isoleucine	A	Adenine
N	Asparagine	C	Cytosine
D	Aspartic Acid	G	Guanine
E	Glutamic Acid	U	Uracil
G	Glycine	B	Exclude A
H	Histidine	D	Exclude C
Q	Glutamine	H	Exclude G
V	Valine	V	Exclude U
[SKA]	S or K or A	N	Any

Table 2: Distribution of Hemagglutinin Amino Acid Sequences Across Subtypes of Influenza A Viruses in GenBank

Subtypes	N1	N2	N3	N6	N7	N8	N9
H1	**30866**	**3711**	0	0	0	0	0
H2	0	**214**	0	0	0	0	0
H3	0	**41009**	0	0	0	0	0
H5	**2343**	0	0	**514**	0	0	0
H6	**391**	0	0	0	0	0	0
H7	0	**264**	**809**	0	**391**	0	**1240**
H9	0	**8636**	0	0	0	0	0
H10	0	0	**156**	0	**686**	**192**	0

type B, which implies its potential utility in designing vaccines against influenza type B. On the other hand, our study did not integrate NA amino acid sequences. Nonetheless, our tree analysis revealed further subdivision of subtypes into smaller groups based on their respective NAs, exemplified by H7N2, H7N3, H7N7, and H7N9 (Figure 2). This finding underscores the subtle diversity within identical HA subtypes. Consequently, additional research and analysis are needed to investigate the potential correlation between the presence of neuraminidase and diversity within the same HA subtype.

Over the past two decades, H5N1, a highly pathogenic avian influenza (HPAI) virus, has circulated annually in Vietnam, resulting in significant economic losses and a high fatality rate[8]. Additionally, the transmission of H1N1 from swine to humans sparked an outbreak in Vietnam during 2009–2010 [10,25]. Consequently, the identified CRs hold promise for developing a universal mRNA vaccine against the prevailing strains of influenza type A in Vietnam.

On the surface of influenza type A virus (IAV), the presence of HA enables its binding to sialic acid on host cell membranes, facilitating viral entry and promoting host infection—thus elevating virus virulence. HA, characterized by the highest rates of evolutionary change among influenza proteins, is unique for each influenza subtype but still retains essential elements of its protein structure[4]. NA is another surface protein of influenza, but due to the high level of antigenic drift[26], HA has become the main target for vaccine design against IAV. The mature form of the HA

Table 3: Hemagglutinin amino acid sequence alignment and epitope prediction against HLA-A*11:01:01, HLA-B*15:01 and HLA-C*07:02

Allele	Strains	Start	End	Peptide	Binding score	Conserved
HLA-A*11:01	H1N1 H1N2 H6N1	385	394	STIDGITNK	0.8921	Yes
HLA-A*11:01	H2N2 H3N2	403	412	KTNEHQIEK	0.7654	Yes
HLA-A*11:01	H2N2 H3N2	500	508	GTYDHDVYR	0.93874	Yes
HLA-A*11:01	H5N1 H5N6 H9N2	28	38	STVDTIMEK	0.90488	Yes
HLA-A*11:01	H5N1 H5N6 H9N2	387	395	KAIDGVTNK	0.9604	Yes
HLA-A*11:01	H7N2 H7N3 H7N7 H7N9	382	390	SAIDQITGK	0.9160	Yes
HLA-A*11:01	H10N3 H10N7 H10N8	383	391	AAIDQITGK	0.9275	Yes
HLA-B*15:01	H1N1 H1N2	369	377	HQNEQGSGY	0.91802	Yes
HLA-B*15:01	H1N1 H1N2	120	128	EQLSSVSSF	0.85285	No
HLA-B*15:01	H7N2 H7N3 H7N7 H7N9	260	268	FIAPDRASF	0.90967	No
HLA B*15:01	H10N3 H10N7 H10N8	211	219	SISVGSSTY	0.89382	Yes
HLA B*15:01	H10N3 H10N7 H10N8	434	442	YQAELLVAM	0.83483	Yes
HLA-C*07:02	H1N1 H1N2	174	182	SYPKLSKSY	0.68198	No
HLA-C*07:02	H5N1 H5N6	463	471	LYDKVRLQL	0.631908	No
HLA-C*07:02	H7N2 H7N3 H7N7 H7N9	154	162	FYAEMKWLL	0.547001	Yes
HLA-C*07:02	H7N2 H7N3 H7N7 H7N9	237	245	GRIDFHWLM	0.590094	No

glycoprotein manifests as a homotrimer with three HA monomers. Each monomer consists of a globular head and a stalk region[27]. Previous studies have shown that the receptor binding site in the globular head is the most variable region and prone to rapid antigenic drift, while the underlying stalk experiences less selective pressure from the immune system[23,27] consequently, this site remains highly conserved not only within but also across HA subtypes belonging to the same group[27]. In our study, by employing an immunoinformatic approach, CRs were also found to be located in the stalk domain. This conservation suggests the potential use of these CRs in stalk-directed strategies for vaccine design.

One strategy to enhance antibodies targeting the immune subdominant stalk region involves the removal of the immunodominant head domain, resulting in a "headless" HA. Stalk-targeting antibodies play a crucial role in inhibiting complement-dependent lysis and antibody-dependent cell-mediated cytotoxicity, effectively halting the spread of viruses[26]. Typically, these antibodies interact with the antigen by attach-

Table 4: Hemagglutinin CRs of influenza type A against HLA-A*11:01 alleles

Allele	Annotati	Strains	Start	End	Binding score	Epitope	mRNA
HLA-A*11:01	CR 1	H1N1 H1N2 H6N1	385	394	0.8921	STIDGITNK	5'_UCN-ACN-AUH-GAY-GGN-AUH-ACN-AAY-AAR_3'
HLA-A*11:01	CR 2	H5N1 H5N6 H9N2	387	395	0.9604	KAIDGVTNK	5'_AAR-GCN-AUH-GAY-GGN-GUN-ACN-AAY-AAR_3'
HLA-A*11:01	CR 3	H2N2 H3N2	403	412	0.7654	KTNEHQIEK	5'_AAR-ACN-AAY-GAR-CAY-CAR-AUH-GAR-AAR_3'
HLA-A*11:01	CR 4	H7N2 H7N3 H7N7 H7N9	382	390	0.9160	SAIDQITGK	5'_UCN-GCN-AUH-GAY-CAR-AUH-CAN-GGN-AAR_3'
HLA-A*11:01	CR 5	H10N3 H10N7 H10N8	383	391	0.9275	AAIDQITGK	5'_GCN-GCN-AUH-GAY-CAR-AUH-CAN-GGN-AAR_3'
Consensus amino acid/mRNA sequence						[SKA]-[TA]-[IN]-[DE]-[GHQ]-[IVQ]-[IT]-[GNE]-K	5'_DMN-RCN-AWH-GAN-SRN-VWN-MHN-RRN-AAR_3'

ing to open hydrophobic pockets within the HA stalk. Notably, the majority of stalk-binding antibodies interact with HA in a heavy chain-directed manner, where hydrophobic residues from an extended HCDR (usually, but not always, HCDR2 or 3) engage with hydrophobic residues from HA to occupy a hydrophobic pocket[26]. Researchers demonstrated that introducing mutations to replace solvent-exposed hydrophobic patches with polar residues stabilized the stem domains of both group 1 and group 2 HA molecules[28]. The removal of the globular head exposed these regions[28]. In 1983, Graves and his team successfully removed the globular head of HA using chemicals, eliciting specific antibodies against the stalk region[26]. However, the efficacy of these vaccines is relatively low, as they exhibit no virus-neutralizing activity due to the denaturation of conformational stalk epitopes[26]. Since then, various strategies have been employed to express and maintain the HA stalk region, such as achieving a neutral-pH conformation. Modifications involving mutations to alter the pH of the stalk from low to neutral have been applied to the H1 and H3 proteins[29,30]. In mouse models, a stable HA stalk antigen known as "mini-HA," based on the H1 subtype antigen, was designed and provided complete protection against both heterologous H1 and heterosubtypic H5 influenza viruses[31]. Postinjection, the serum from these mice produced antibodies that bound to a range of HAs, competed with human broadly neutralizing antibodies for binding to the HA stem, impeded the spread of H5N1 viruses, and facilitated antibody-dependent effector activity.

In addition to removing the globular head of HA, an alternative approach to inducing antibody reactivity to the stalk involves the observation that sequential exposure to influenza viruses with different heads but conserved stalk domains redirects the immune response toward the normally subdominant stalk. This strategy is implemented through vaccination with chimeric HAs, where these chimeric constructs comprise H1 (group 1) or H3 (group 2) stalk domains combined with 'exotic' head domains, usually of avian origin[32]. The head domain is distinguished from the stalk domain by a conserved disulfide bond formed between cysteines 52 and 277. The segment of the HA ectodomain located between these amino acids constitutes the head domain, while the remaining part is identified as the stalk domain (N- and C-terminus

of HA1 and the ectodomain of HA2). Structural epitopes in the stalk domain can be stabilized by incorporating a heterologous head domain. Importantly, unlike many headless HA approaches, the stalk in chimeric HAs is correctly folded and fully functional. In fact, influenza viruses expressing chimeric HAs can be rescued and cultivated to titers comparable to those of wild-type HAs in both embryonated eggs and cell culture by employing various methods[32]. Subsequent studies demonstrated that sequential vaccination with different chimeric HAs sharing the same stalk but displaying different heads induces high titers of stalk-reactive antibodies in mice and ferrets. Furthermore, it provides broad protection against lethal challenges with various group 1 and group 2 viruses, including H5N1 and H7N9 viruses, achieved through the administration of vaccines in a specific order (33). Animals vaccinated with group 1 constructs did not develop titers against group 2 stalks, making them susceptible to group 2 (H3N2) virus challenge. This suggests that a successful chimeric HA-based vaccine should include three components: a group 1 component, a group 2 component, and an influenza B chimeric HA component[33].

Chimeric HA-based vaccine approaches have been successfully implemented in the form of DNA vaccines, recombinant protein vaccines, and viral vectors. These approaches have been coupled with various experimental adjuvants, including oil-in-water emulsions similar to those licensed for use in humans[33,34]. Furthermore, a phase 1 clinical trial involving a chimeric HA (cHA)-based vaccine has been reported[35,36]. Participants received two vaccinations, each comprising different combinations of a chimeric HA live attenuated virus vaccine, an inactivated virus vaccine, or an inactivated virus vaccine combined with an adjuvant. The vaccination successfully induced anti-stalk H1 antibodies, demonstrating both safety and efficacy. Notably, participants who received two doses of a chimeric HA-inactivated adjuvanted virus maintained anti-stalk antibodies at a level at least four times higher than the baseline for up to one and a half years, yielding optimal results. With subsequent exposure to recombinant H1 stalk virus, mice that received a passive transfer of sera from vaccinated participants exhibited a trend toward reduced weight loss compared to mice that received sera from the placebo group, despite both groups receiving sera. This clinical trial lends support to the ability of the chimeric HA vaccine strategy to induce anti-stalk antibodies in humans. However, additional clinical trials are necessary to demonstrate the effectiveness of these stalk-directed antibodies in protecting humans from infection. While H1 subtype vaccines have been the primary focus of research and development efforts, this approach has also been explored for H3[37] and influenza B[38], yielding encouraging outcomes. Currently, it remains to be investigated whether these candidate immunogens can be combined into a multivalent vaccine.

In addition to the "headless" HA and cHA-based vaccines, the "mosaic" HA vaccine is another stalk-directed strategy for vaccine design. Replacing the immunodominant antigenic sites on the HA1 head to produce a "mosaic" HA1 region is an additional method that can be utilized to suppress the immunodominance of the HA1 head subunit and shift the immune response to the stalk region. The amino acid sequences taken from an avian HA protein (either H10 or H14) were used to replace the immunodominant antigenic sites of the H3 head with[39]. This method was successful in silencing those sites. Compared to a commercially available inactivated seasonal vaccine, the ability of the recombinant mosaic H3 protein vaccine to induce H3 stalk-directed antibodies in mice was superior. Mice that had been vaccinated with an adjuvanted mosaic recombinant H3 protein were protected from death when they were subjected to a lethal challenge with two different heterologous H3 viruses but still experienced severe weight loss of at least 20%[39].

Despite the potential of stalk-directed approaches for inducing cross-reactive immunity against the continuous mutation of influenza type A, these strategies still face significant obstacles. The moderate immunogenicity of the stalk domain necessitates up to three vaccinations to successfully induce this immunity. While animals are protected from death after being challenged with homosubtypic strains, they often experience severe morbidity, losing more than 10% of their weight, especially in mouse models[39]. This is likely due to the Fc-mediated mechanism of action, which is a substantial contributor to protection in many stalk-directed strategies. However, this mechanism does not neutralize the virus; instead, it contributes to viral clearance after infection[32]. Thus, the robustness of protection is diminished with these stalk-directed approaches, despite an increased breadth of reactivity[40]. This poses a significant concern for further clinical trials in humans.

Additionally, the stalk region of HA remains susceptible to selective pressures from the immune system, despite being more conserved than the head region of HA[41]. This is evident when selecting CRs at a consensus threshold of 80%, as variability within subtypes persists. Notably, preexisting stalk-directed antibody

titers after human influenza challenge have been reported to select for a stalk-antibody escape mutant[40]. Furthermore, there is no consensus on the efficacy of these anti-stalk antibodies in human patients in the scientific literature. While one study using a human household transmission experimental design revealed a correlation between HA stalk-directed antibodies and protection from infection[42], another study using human samples showed no significant correlation after adjusting for head-specific antibodies[43]. Adjustments were made to account for antibodies specific to the head. Moreover, a phase II trial examining the therapeutic efficacy of a monoclonal stalk-directed antibody revealed no clinically significant effect on influenza disease[44]. These contradictory results raise questions about the protective efficacy of these anti-stalk antibodies in human patients.

On the other hand, careful consideration must be given to the population coverage of T lymphocyte epitopes in vaccine design. The presence of more than a thousand human MHC alleles worldwide poses a significant challenge, as only individuals with a specific MHC allele recognizing the epitopes in our vaccine construct will mount an immune response upon exposure to the vaccine. In our study, the CRs exhibited the highest affinity for the HLA A*11:01 allele, which is one of the most frequently occurring alleles among the Kinh Vietnamese population[15]. Consequently, they hold promise for effectively triggering herd immunity in the Vietnamese population.

Compared with time-consuming and expensive laboratory-based methods, utilizing an immune-informatics approach provides the advantages of cost savings, rapid epitope identification and screening, and efficient vaccine design. Technologies that enable swift and high-yield production, such as the development of mRNA vaccines, offer a promising solution against the continuous mutation of IAVs. Our immune-informatics-based approach for designing a universal mRNA vaccine targeting the stalk domain of HA molecules in influenza-type viruses has yielded promising results. These novel vaccine candidates have potential for use in the development of a universal vaccine against IAVs. On the other hand, it is crucial to note that our study specifically focused on stimulating the binding of epitopes against T-cell MHC class I molecules. However, a comprehensive evaluation of mRNA sequences should consider factors such as toxicity, allergenicity, and cytokine inducibility for both T-cell and B-cell responses. Additionally, beyond the mRNA sequences encoding the epitopes, the presence of four primary elements in the open reading frame (ORF) is essential for constructing a highly immunogenic mRNA vaccine: 1. Kozak sequence; 2. Adjuvant; 3. Linkers (or spacers); 4. Stop codon. To enhance the efficacy of vaccine design, further research should concentrate on the currently circulating IAV strains within the Vietnamese population.

Finally, despite advancements in mRNA vaccines, this technique has inherent limitations. The low stability and susceptibility to degradation of mRNA molecules present challenges that necessitate solutions[45]. Consequently, critical factors such as manufacturing, quality control, formulation, immunological and physicochemical properties of the vaccine, and the translated form of the peptide remain significant challenges in the development of mRNA vaccines.

CONCLUSION

The analysis of HA amino acid sequences from 15 human-infected influenza A virus strains obtained from the WHO IVTM database suggested a high predominance of the H1N1 and H3N2 subtypes as key contributors to seasonal flu. With 82,200 cleaned and suitable sequences, they formed a solid foundation for further epitope prediction and conservation assessment. The integration of T-cell epitope prediction, MHC class I binding analysis, and multiple sequence alignment identified 16 common epitopes across diverse influenza A virus strains. Through meticulous evaluation, 11 of these epitopes were revealed to be conserved, with the five most antigenic sequences (CRs) located in the less antigenically variable stalk domain (HA2). These findings present an opportunity to design an HA stalk-directed universal mRNA vaccine targeting multiple influenza strains. Furthermore, the phylogenetic analysis confirmed the distinct groupings of 15 HA subtypes, with each conserved region effectively covering its representative strains. These findings, particularly the potential coverage against influenza type B by CR3, suggest a viable strategy for developing a broadly effective mRNA vaccine against prevalent strains, such as those observed in Vietnam.

LIST OF ABBREVIATIONS

HA Hemagglutininc

HA Chimeric Hemagglutinin

NA Neuraminidase

MHC Major histocompatibility complex

CRs Conserved regions

HCDR Heavy chain complementary determining region

COMPETING INTERESTS

The author(s) declare that they have no competing interests.

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