Original Article

Design, Synthesis and *In Vivo* Evaluation of a Candidate Fusion Epitopic Construct Vaccine Based on M2e, HA1, HA2, NA and NP Fragments of the Highly Pathogenic Avian H5N1 Influenza Virus

Hamidi, A[1], Farzin, H[2], Haghparast, A[1,3*]

1. *Biotechnology Section, Department of Pathobiology, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran*
2. *Razi Vaccine and Serum Research Institute, Agriculture Research, Education and Extension Organization (AREEO), Mashhad, Iran*
3. *Immunology Section, Department of Pathobiology, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran*

How to cite this article: **Hamidi A, Farzin H, Haghparast A. Design, Synthesis and *In Vivo* Evaluation of a Candidate Fusion Epitopic Construct Vaccine Based on M2e, HA1, HA2, NA and NP Fragments of the Highly Pathogenic Avian H5N1 Influenza Virus. Archives of Razi Institute Journal. 2024;79(4):849-856.** DOI: 10.32592/ARI.2024.79.4.849

Article Info:
Received: 24 April 2024
Accepted: 27 May 2024
Published: 31 August 2024

Corresponding Author's E-Mail:
alireza.haghparast@gmail.com
haghparast@um.ac.ir

ABSTRACT

The H5N1 subtype of the influenza virus is highly pathogenic and lethal to humans and animal. The necessity for the development of new vaccines with a broad spectrum of efficacy against this pathogen seems to be very crucial. One highly regarded solution to this problem is to design and production of recombinant vaccines using the conserved peptide of influenza viruses. A search of international databases yielded the peptide sequence of the M2e fragment of H5N1 viruses isolated from Iran, as well as a variety of conserved peptide sequences of fragments of HA1, HA2, NA and NP of other H5N1 viruses. These sequences were obtained for both MHC receptors in mice. Subsequently, these fragments, in conjunction with a PADRE sequence, were connected by bioinformatics to design a fusion epitopic construct. Subsequently, the construct was optimized for expression in *E.coli* BL21. Following the expression and purifications of the fusion epitopic construct, it was injected subcutaneously (SC) into the hindlimb muscles of 6–8 old week female BALB/c mice. Three weeks following the conclusion of the second immunization, the mice in both immunized and control groups were weighed and checked for any adverse effects at the injection sites. Subsequently, the mice were euthanized and blood was collected from their hearts to determine the total IgG antibody titer before and after immunization by ELISA. No evidence of local inflammation or complications was observed at the SC injection sites until the end of the experiment. Additionally, the autopsy of mice showed no bleeding or lesions in organs, particularly the liver and spleen. The mice exhibited no significant change in weight throughout the immunization period. The total IgG level, as determined by average OD value in the serum of immunized mice, was found to be five times higher (5.881 ng/ml) than that of the control group (1.143 ng/ml). The results demonstrated a highly significant IgG antibody response following SC administration of an immunogenic recombinant peptide in mice.

Keywords: Influenza Virus, H5N1, Fusion Epitopic Construct, Vaccine, IgG

1. Introduction

Since the initial influenza pandemic, influenza viruses have remained the most important respiratory pathogen in humans and one of the most critical pathogens in the livestock and poultry industry (1). It is estimated that between 10 and 20 percent of the world population is affected by seasonal influenza epidemics every year. Of these, between three and five million individuals experience severe disease and 250,000 to 500,000 die yearly (2). In the livestock and poultry industry, infection from influenza viruses causes significant damage every year, which poses a considerable risk to the global health system (3). The classification of influenza viruses is within the *Orthomyxoviridae* family and include four genera: Alpha, Beta, Gamma, and Delta influenza viruses, or A *Influenzavirus*, B *Influenzavirus*, C *Influenzavirus*, and D *Influenzavirus* (4). Of the four genera, only types A, B and C have been demonstrated to be pathogenic in humans, while the pathogenicity of genus D has never been reported in humans (4). The most crucial group of influenza viruses is Type A, which is classified into 198 subtypes based on two types of surface proteins: Hemagglutinin (HA) and Neuraminidase (NA). The genome of Type A influenza virus is a single-stranded RNA (ssRNA), negative sense, fragmented into eight segments (4). Recombination between these segments is responsible for the emergence of new influenza virus subtypes during the replication process. In addition, spot mutations of influenza viruses contribute to the development of resistance to vaccines and drugs, with a high prevalence (4). Currently, all influenza vaccines use the viral HA protein to elicit immunity during infection. he traditional method of producing the influenza vaccine involves culturing the virus in hatched eggs. However, other methods are also being developed, including the cultivation of the virus in cell lines and the expression of antigenic fragments by a range of other organisms, including viruses, bacteria, yeasts, algae, and plants (5, 6). Given the considerable number of influenza virus subtypes that are currently in circulating among humans and animals, influenza vaccines are generally available in trivalent or quadrivalent vaccine formulations. However, due to the unpredictable nature of the subtypes responsible for the influenza virus pandemics and the mutations in the HA protein, the effectiveness of these vaccines is limited, and they are only usable for six months (7). In contrast to the HA protein of influenza viruses, which is highly variable and undergoes frequent mutation, genomic areas have been identified in influenza viruses that are highly conserved. These areas can be used to design and develop vaccines which are potentially more effective and induce long-lasting protective immunity in the host (8). The most notable of these regions is the outer piece of the protein matrix two, denoted as M2e, which exhibits a striking degree of conservation across diverse influenza virus strains. The 23 amino acids comprising the M2e sequence display remarkable uniformity, with minimal variation observed across different influenza viruses. One of the different influenza virus subtypes that is commonly found in both humans and animals and can cause severe disease and high human mortality rates in humans is the H5N1 subtype. This subtype is classified as HPAI (High Pathogenic Avian Influenza) in birds. According to the World Health Organization (WHO), this subtype may potentially cause an influenza pandemic in humans in the future. The most advanced technology for the preparation of influenza vaccines is the production of immunogenic peptide components of viruses in microorganisms using recombinant DNA technology. In recent years, there has been a great deal of interest and attention directed towards the utilization of conserved epitopic sequences of antigenic proteins of influenza viruses. Matrix protein 2 (M2e) sequences and peptide components derived from nucleoprotein (NP) and neuraminidase (NA) proteins of the influenza virus can be used to generate recombinant peptide vaccines that elicit more significant and more prolonged immunity and effectiveness (9). Therefore, in the present study we designed and developed a multi-epitopic recombinant peptide vaccine based on M2e, HA1, HA2, NA and NP proteins of H5N1 subtype and evaluated its immunogenicity in BALB/c mice.

2. Materials and Methods

2.1. M2e Sequence Selection

A search of the flu virus databases including GISAID, BV-BRC and NCBI revealed that, it was found that so far seven strains of HPAIV H5N1 influenza virus isolated from swans, chickens and ducks from Iran between 2006 and 2017 have been registered in the global flu virus databases. However, only three of the strains contain the M2 gene sequence, specifically the region between amino acids 2 and 24 of the M2 protein, which is commonly referred to as the M2e region. The M2e sequences of three Iranian H5N1 influenza viruses were aligned using the T-COFFEE program (Barcelona, Spain). It was found that the two sequences exhibited complete similarity, while the third sequence differed by a single amino acid. Furthermore, the sequences were also aligned with the M2e sequences of the four international vaccine strains of the H5N1 virus. It was found that the amino acid sequences of two Iranian strains and three vaccine strains were completely identical. Accordingly, the M2e sequence of Iranian H5N1 strains exhibited 100% similarity to H5N1 vaccine strains was employed in this study (Figure 1).

2.2. The Selection of Conserved H5N1 Epitopic Sequences from HA1, HA2, NA, and NP Proteins with High Affinity Binding to Mice MHC Class I and II Molecules

The conserved epitopic sequences were derived from HA1, HA2, NA, and NP proteins of the H5N1 influenza virus and were selected from the IEDB and BV-BRC databases for both MHC class I and II receptor types in mice, which are Kd (equivalent to MHC I) and IAd (equivalent to MHC II) (Table 1).

2.3. Design and In Silico Computational Analysis of Fusion Epitopic Construct Vaccine

The amino acid sequences of the selected epitopes that are to be used to design the vaccine must bind together, and this requires the use of amino acid sequences called linkers, which are different for each epitopic receptor. The linker sequences corresponding to the H5N1 peptide are listed in Figure 2. The amino acid sequence of the epitopic fragments and linkers were edited using the SnapGene program (version 5, Boston, USA),followed by the ProtParam program (version 10, Boston, USA) to examine the physical and chemical properties needed to peptide expression. It was determined that the designed peptide exhibited the required properties for expression. Peptide DNA sequences designed for expression in *E.coli* bacteria were optimized for codons using the Jcat program (Braunschweig, Germany).The resulting sequence was synthesized for expression. An 18-piece peptide was obtained, consisting of 3 M2e sequences, a PADRE sequence, 7 IAd epitopic sequences, and 7 Kd epitopic sequences.It obtained 307 amino acids with a molecular weight of 32.321 Kilo Daltons (KD) (Figure 2). To identify an appropriate plasmid for the expression of the designed fusion epitopic construct sequences in *E.coli* BL21,the pET28a$^+$ plasmid was selected from the Addgene site (Watertown, USA). The MCS part of the plasmid contains 11 recognition sites for restriction endonuclease enzymes (REs). To ascertain the presence of these sites within the DNA sequence of the peptide construct, the MCS was subjected to analysis using the CLC and NEBCutter programs, developed by CLC (Redwood, USA) and NEBCutter (Ipswich, USA), respectively. It was determined that the sequence does not have any cutting sites for the REs part of the MCS plasmid pET28a$^+$. In the subsequent step, the ORF Finder program was used to examine the presence of the start codon and stop codon in the DNA sequence, and found that this sequence lacked both the start and stop codons, exhibiting a single open reading frame (ORF). The final stage of the DNA sequence was placed inside the pET28a$^+$ plasmid by the SnapGene program. NcoI and XhoI restriction endonuclease (RE)cutting sites were created at the 5' ends, respectively, followed by the addition of the sequence required for the His tag.

2.4. Preparation of Recombinant Plasmid and Clone in Bacteria

The synthesis of fusion epitopic DNA sequences and clones in the pET28a$^+$ plasmid was carried out by Biomatic (Lincoln, Canada). The bacterial strain BL21 *E.coli* was obtained from NEB (Ipswich, USA). The antibiotic Kanamycin which is required for plasmid transformation screening, was procured from Sigma-Aldrich (St. Louis, USA). The recombinant plasmid, prepared using a Thermo Scientific transformation kit (Waltham, USA), was introduced into *E.coli* and the Blue-White screening method was used using X-gal, IPTG, and Kanamycin (Merck, Germany) for the initial screening of transformed bacteria. The white colonies of transformed bacteria were isolated in a - culture medium containing Kanamycin. To confirm the presence of the recombinant plasmid in these bacteria, a colony PCR method was used using primers designed by the vector NTI program (Version 10, CA, USA) and synthesized by Arian Gene Gostar (Tehran, Iran).The primers used were ATGGCGTCTCTGCTGAC(forward) &CTCGAGCAGAGAAGAAGCAAC(reverse) and the method used was colony PCR.

2.5. Expression, Purification and Quantitative Measurement of Fusion Epitopic Construct

To express the fusion epitopic construct, 50 milliliters of Terrific broth culture medium was used to incubate 50 ml of E. coli BL21 bacteria containing a recombinant plasmid overnight at 37°C in a shaker incubator. Subsequently, 1 ml of the bacterial culture was added to another 200 ml of the other terrific broth culture medium and incubated in the 37°C shaker incubator. The following step was to measure the OD of the bacterial culture sample a wavelength of 600 nm at one-hour intervals. Once the OD value reached 0.5 to 0.8.IPTG was added to the culture medium inside the shaker incubator and incubation was continued up to 4 hours to allow the peptide expression. Finally, the bacterial culture was removed from the incubator and subjected to centrifugation at 10,000 rpm for 5 minutes at 4°C. The bacterial pellet was lysed using a lysis buffer solution containing Phenyl Methyl Sulfonyl Fluoride (PMSF), which inhibits proteases to extract the fusion epitopic construct. This was done using a Hielscher homogenizer (Teltow, Germany) and IKA sonication (Staufen, Germany). Subsequently, the samples were subjected to centrifugation the previously established conditions, and the resulting supernatant containing the extracted proteins was isolated and filtered through a 0.2-micron Millipore filter. The fusion epitopic construct was purified using a protein extraction kit with nickel columns (ABT Affinity His-Tag Ho Chi Minh, Vietnam). The purified fusion epitopic construct subjected to examination via Nanodrop (Thermo Scientific Waltham, USA) to check the extraction quality at wavelengths 340 and 280 nm. As for the extract in BCA (Bi Cinchoninic Acid) protein a quantitative measurement kit (Pars toss, Tehran, Iran) was used to measure the final peptide concentration. Then, the SDS-PAGE method was used to determine the molecular weight of the extracted peptide. Finally, the Western blot method was used to confirm of fusion epitopic construct with anti-His-tag antibody by DAB (Di Amino Benzidine) staining.

2.6. Immunization with a Fusion Epitopic Construct in the BALB/c Mice

The 6-8 old week female BALB/c mice (Razi, Mashhad, Iran) were divided into two groups: a control group and an immunized group. Each of these groups consisted of six mice. On the day of immunization, 20 µg of the fusion epitopic construct was dissolved in 100 µl of sterile normal saline prepared with nonpyrogenic injectable distilled water and injected via the SC route into the upper hindlimb

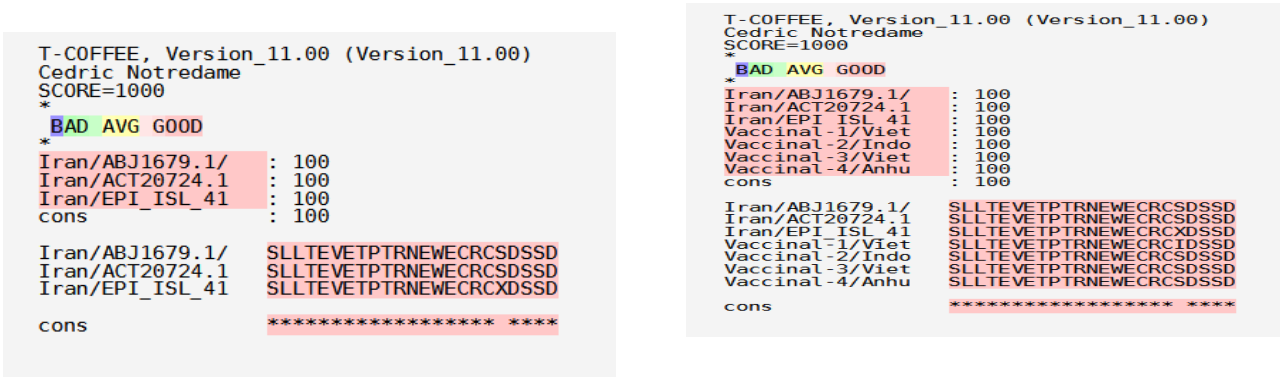

Figure 1. Amino acid sequence alignment of M2e sequence of three Iranian strain H5N1 virus (left panel) and the results of the alignment of Iranian M2e sequences present in vaccine strain (right panel)

Table 1. Selected epitopic sequences from HA1, HA2, NA and NP proteins of H5N1 virus with high affinity for the mice Kd (MHC I) and IAd (MHC II) receptors

Receptor Kd (MHC I)			Receptor IAd (MHC II)		
Amino acid sequence	**Epitope type**	**Protein origin**	**Amino acid sequence**	**Epitope type**	**Protein origin**
LYQNPTTYI	predicted	HA1	DCTLIDALLGDPH	Experimental	HA1
IYSTVASSL	experimental	HA2	HNTNGVTAACSHE	Experimental	HA1
SSFSVKQDI	experimental	NA	STNQEQTSLYVQA	Experimental	HA2
TYQRTRALV	experimental	NP	STNQEQTSLYVQAS	Experimental	HA2
			NFLTEKAVASVTLAG	Predicted	NA
			TELKLSDYEGRLIQNS	Experimental	NP

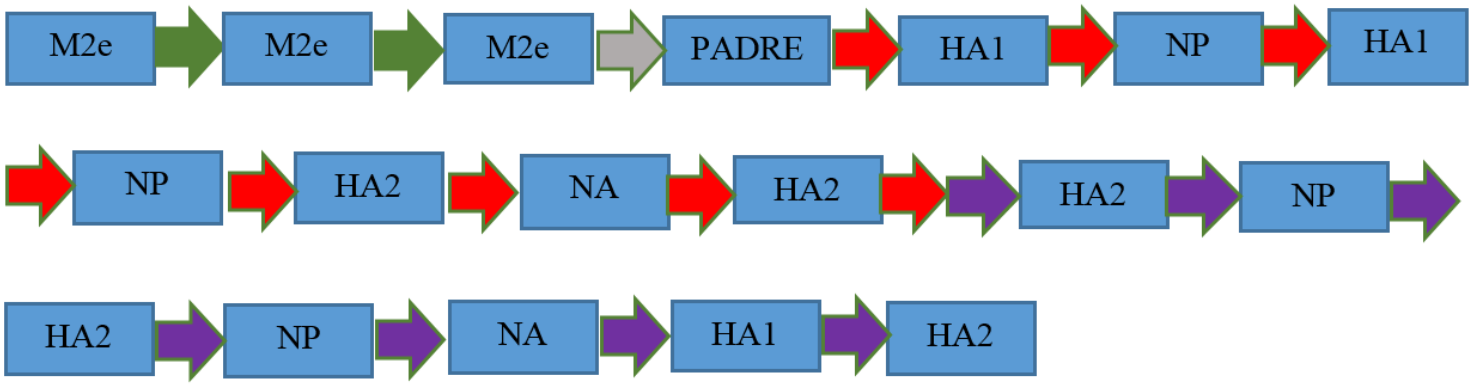

Figure 2. Final design of the fusion epitopic peptide construct

muscles of the immunized group by an insulin syringe. The control group was administered mice of the were injected with only 100 µl of normal saline. Booster immunizations were performed 14 days after the first immunization. Three weeks after completion of the second immunization, both groups of mice were weighed and examined for any side effects at the injection sites. Then, the mice were euthanized by inhalation of high concentration of CO_2 gas in a special cage and blood was collected from their heart using a 2 ml syringe.

2.7. Measurement of Total Serum IgG Antibody Levels After Fusion Epitopic Construct Immunization

The total Mouse IgG kit (Cygnus Technology F049, NC, USA) was used to determine the total IgG antibody in the blood of mice after fusion epitopic peptide immunization. The kit has a detection accuracy of 0.3 ng/ml. A total of 50 µl of each test serum sample and standard control solution were utilized. The kit contains five dilutions of 0, 0.25, 1, 4 and 20 ng poured into each well. The procedure was conducted in accordance with the instructions provided in the kit ,and the results were read in two wavelengths (405 and 492 nm) using an ELISA reader (Biotek, Vermont, USA). The average absorption was then calculated at two wavelengths, and using the standard curve obtained from the control solution, the amount of total IgG antibodies in the serum of the mice was obtained in ng using the standard curve obtained from the control solution.

2.8. Statistical Analyses

The statistical analysis was performed using the GraphPad Prism program (version 10, Boston, USA). An unpaired t-test was used to examine the meaningful difference between the immunized and control groups with a P value < 0.0001 was considered statitistically significant.

3. Results

3.1. H5N1 Fusion Epitopic Construct Has the Right Size, Purity, Accuracy and Quantitative Measurement

The result of the proprietary primer PCR colony result confirmed the DNA sequence of the fusion epitopic construct fragment with a length of 921 bp. This was evident from the presence of a band in the range between 900 bp and 1000 bp in all PCR samples. The SDS-PAGE of the purified fusion epitopic construct showed a band at approximately 32 KD, confirming the production of the fusion epitopic construct by bacteria. Also, the Western blot result of the purified fusion epitope construct demonstrated the presence of a specific band, indicative of successful binding between DAB and the anti His-tag antibody. The BCA quantitative measurement of the purified fusion epitopic construct peptide yielded a result of 0.455 mg/ml, which equates to 455 µg/ml (Figure 3). This indicates that the amount of fusion epitope construct peptide produced is 0.455 mg/ml equal , or 455 µg/ml (Figure 3).

3.2. SC Immunization with H5N1 Fusion Epitopic Construct in Mice Is Safe Without Any Weight Loss and Detrimental Side Effects

Following the administration of the fusion epitopic construct to mice twice, no adverse effects were observed until the end of the experiment. No local complications were observed at the SC injection sites until the end of the experiment. The autopsy of the mice showed no evidence of bleeding or macroscopic lesions in the liver or spleen (data not shown). The weight of the mice remained relatively consistent throughout the immunization period (Table 2).

3.3. H51N1 Fusion Epitopic Construct Immunization Induces High IgG Levels in BALB/c Mice

Following two rounds of immunization, blood samples were collected from the hearts of the mice seven weeks after the start of the experiment. The serum samples were then separated for analysis. An ELISA was performed to measure the total IgG levels in the serum samples of the immunized and control mice. The OD values were converted to nanograms (ng) in accordance with the instructions provided in the kit. The average IgG value in the control group was 1.143 ng/ml, while the average in the immunized group was 5.881 ng/ml. This represents a significant increase of more than five times the IgG antibody in the immunized group compared to the control group. The significant differences and normality of the results obtained from the two groups were calculated and analyzed using an unpaired t-test statistical method, with a and P value < 0.0001 (****), which was considered significant (Figure 4).

4. Discussion

The World Health Organization (WHO) has indicated that the H5N1 virus may be the cause of a future influenza pandemic with high human casualties in the future (11). It is therefore vital that a new generation of vaccines with effective and protective features is developed as a matter of urgency in order to limit the number of casualties and infection rate caused by this virus (10, 11, 12). The high risks associated with of the H5N1 subtype make it challenging to develop a vaccine using cultivation methods in eggs and cell lines that require specialized protective equipment such as BSL3 (Bio Safety Level 3) laboratories (13). To address this challenge, the expression of viral immunogenic proteins in microorganisms, including bacteria, viruses, yeast, algae, plants and animals can be used to develop practical vaccine components (6, 14). The M2 protein of the influenza H5N1 virus is made of seven gene fragments and contains 97 amino acids. This protein is tetrameric and located on the surface of the lipid envelope of the virus. It consists of three parts: 1. Region N of the extracellular terminal (M2e-amino acids 2 to 24), 2. Region TM (amino acids 25 to 46), and 3. Region C is the intracellular terminal (amino acids 47 to 97). By creating an ion channel in the virus membrane, the M2 protein causes the ion (H^+ or Proton) to enter the virion from the acidic space of the endosome and initiate the process of uncoating the influenza virus and entry of the ribonucleoprotein (RNP) of the virus into the host cell and initiate infection (4).

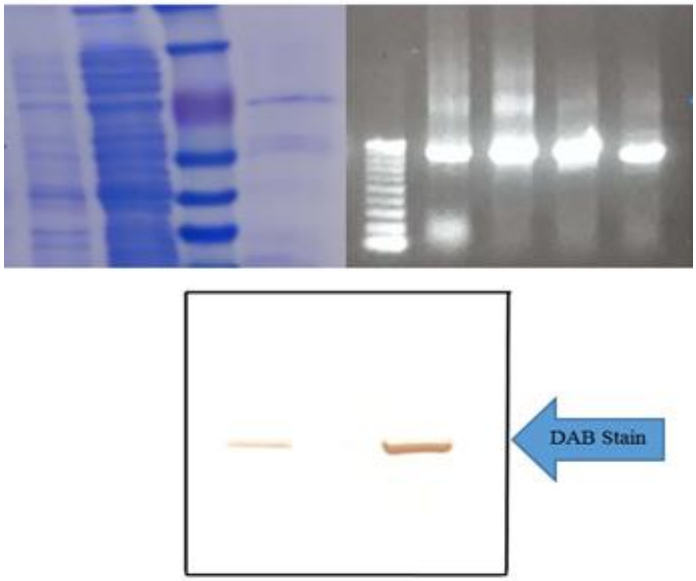

Figure 3. Colony PCR of transformed bacteria (top right panel) showing the right band (921 bp), SDS-PAGE of purified fusion epitopic peptide construct (top left panel) showing the right band (32 KD), and Western blot analysis of purified fusion epitopic peptide construct (lower panel)

Table 2. Weight changes of mice in immunization group during the experiment

No of Mice	Start of Immunization	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7
1	45 gr	46 gr	45 gr	44 gr	46 gr	46 gr	47 gr	44 gr
2	47 gr	45 gr	46 gr	47 gr	47 gr	46 gr	48 gr	48 gr
3	39 gr	40 gr	41 gr	40 gr	39 gr	40 gr	39 gr	38 gr
4	40 gr	41 gr	40 gr	41 gr	39 gr	41 gr	40 gr	41 gr
5	43 gr	42 gr	43 gr	44 gr	43 gr	44 gr	42 gr	43 gr
6	38 gr	39 gr	40 gr	39 gr	39 gr	41 gr	40 gr	39 gr

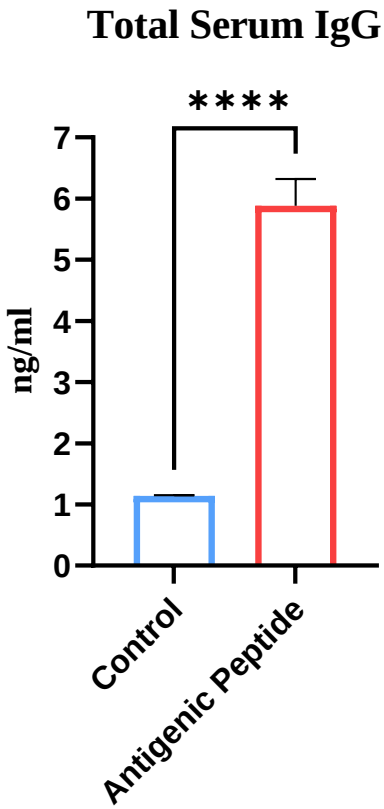

Figure 4. Comparison of total IgG antibody levels between immunized and control mice after SC injection of fusion epitopic peptide construct immunization measured by ELISA. The significant difference between the two groups were calculated using unpaired t-test.

The humoral and cellular immunity of anti-M2e antibodies against influenza viruses is carried out through several mechanisms, including:
1. Prevention of the budding and release of replicated viral particles from infected cells, **2.** Antibody-mediated cellular cytotoxicity (ADCC), **3.** Cytolysis mediated by complement and 4. Antibody-mediated phagocytosis (15). Given the significance of the M2e protein in viral immunity and theH5N1 virus's pathogenesis, as well as the presence of conserved regions in this protein, our objective was to develop a candidate immunogenic multiepitopic peptide construct to induce immunity in BALB/c mice. In the present study, we have employed a strategy involving three consecutive pieces of M2e protein with the objective of enhancing the immunogenic capability against this protein. In addition to this, we also used 14 peptide sequences of H5N1 virus conserved epitopes present in HA1, HA2, NA and NP proteins. The epitopic sequences were organized into two proportional groups to bind to Kd (equivalent to MHC I) and IAd (equivalent to MHC II) receptors in mice. To increase the immune system's ability to produce the required antibodies, a piece called PADRE, a 13 amino-acid- sequence of the pan-epitope MHC II in mammals, was used as an internal adjuvant (16,17). In this study we used SC injection, contrary to the routine intramuscular (IM) injections of influenza vaccines. Studies have shown that SC injection of vaccines have greater immunostimulatory effects than IM injection due to the high number of dendritic cells (DCs) present in the SC area, which play a very important role in processing antigens, delivering them to T cells and producing antibodies (18,19). Similar studies by researchers in this field have produced significant results. Mytle and Leyrer (20) designed a multi-epitopic vaccine using M2e components and epitopes of other influenza virus components that could immunize mice against the H5N1 virus. In another study, Kalaiyarasu and Bhatia (21) designed a fusion peptide with M2e and HA2 components of the H5N1 virus, which produced a high value of effective antibodies after injection into the mice. Hendy and Amouzougan (22) showed that influenza vaccines based on M2e and HA proteins of the virus can reduce the effects of disease and mortality in influenza patients in addition to providing protection. Studies have also shown that the use of M2e, along with other conserved epitopes of influenza viruses can create homologous immunity as well as heterologous immunity against other influenza virus subtypes (8, 20, 21, 23, 24, 25). Subbiah and Oh (25) combined M2e and HA components of the H3N2 virus and produced a vaccine that generates specific antibodies and activates CD4 and CD8 cells along with the ADCC process in mice against H1N1, H3N2, H5N1, H7N9 and H9N2 viruses. Kim and Li (24) designed the VLP vaccine using M2e and NA components, which could provide immunity to H1N1, H3N2, H5N1, H7N9, and H9N2, as well as influenza B viruses in mice. On the effects of vaccine strengthening immunity by the PADRE component, Gomes and Zhang (16) indicated that the presence of this component as the MHC II Pan-epitope increases the immunization capability of the M2e-based influenza vaccine. Our study demonstrated the appropriate effect of the fusion epitopic construct vaccine based on M2e fragments and MHC I and II epitopes of the H5N1 influenza virus and PADRE fragment using SC injection method which ultimately led to the significant increase in IgG antibody levels in the immunized mice. The experiment also showed that the designed peptide vaccine and the SC injection lacked local and systemic side effects in the body of immunized mice. To more closely examine the effects of the fusion epitopic construct vaccine in this study, further studies are needed to study the mechanistic effect of increasing the immunity of adjuvants and to examine other immune mediators such as cytokines and effector T cells as well as challenge studies of the immunized mice with wild type influenza viruses.

Acknowledgment
This study was partially supported by a grant from Ferdowsi University of Mashhad. We would like to express our gratitude to the members of the Razi Vaccine and Serum Research Institute of Arak and Karaj, including Dr. Ahmadi, Dr. Ebrahimi, Dr. Ghaemmaghami and Dr. Ranjbar for invaluable assistance in this study.

Authors' Contribution
A.H. was responsible for conducting the experiments, prepared the manuscript, contributed in the design of the study and funding acquisition. H.F. provided invaluable assistance in the design of the study and funding acquisition. A.H. (corresponding author) conceived the idea, designed the study, wrote and edited the manuscript, contributed in funding acquisition and supervised the whole study.

Ethics
The animal study was approved in accordance with the ethical guidelines set forth by the Animal Care and Use Committee of Ferdowsi University of Mashhad (license number 46013-M). It is declared that all ethical considerations were considered in the conducted research and preparation of the submitted manuscript.

Conflict of Interest
The authors confirm that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data Availability
The data that support the findings of this study are available on request from the corresponding author.

References

1. Yamauchi Y. Influenza virus method and protocols. In: Walker JM, editors. School of cellular and molecular medicine, University of Bristol, Bristol, UK. New York: Humana press; USA. 2018. P. 1-22.
2. Herrera-Rodrigueza J, Meijerhofa T, Niestersa HG, Stjernholmb G, Hovdenb AO, Sørensena B, et al. A novel peptide-based vaccine candidate with protective efficacy against influenza A in a mouse model. Virology. 2018;515:21-28.
3. Swayne DE. Animal influenza. Exotic and emerging avian viral diseases research unit. Chennai: Wiley blachwell. India. 2017.P.3-30.
4. Howley PM, Knipe DM. Fields virology: Emerging viruses. In: Whelan S, editors. Department of immunology Harvard medical school. Philadelphia: Wolters Kluwer. USA. P.809-859.
5. Frutos AM, Price AM, Harker E, Reeves EL, Ahmad HM, Murugan V, et al. Interim Estimates of 2023–24 Seasonal Influenza Vaccine Effectiveness United States. Morbidity and Mortality Weekly Report. 2024;73(8):1-7.
6. Zhao G, Du L, Xiao W, Sun S, Lin Y, Chen M, et al. Induction of protection against divergent H5N1 influenza viruses using a recombinant fusion protein linking influenza M2e to Onchocerca volvulus activation associated protein-1 (ASP-1) adjuvant. Vaccine. 2010; 28: 7233–40.
7. Lim CML, Komarasamy TV, Adnan NAAB, Radhakrishnan AK, Balasubramaniam VRMT. Recent Advances, Approaches and Challenges in the Development of Universal Influenza Vaccines. Influenza and Other Respiratory Viruses. 2024;18:1-13.
8. Sheikh QM, Gatherer D, Reche PA, Flower DR. Towards the knowledge-based design of universal influenza epitope ensemble vaccines. Bioinformatics. 2016; 32(21): 3233-9.
9. Tutykhina I, Esmagambetov I, Bagaev A, Pichugin A, Lysenko A, Shcherbinin D, et al. Vaccination potential of B and T epitopeenriched NP and M2 against Influenza A viruses from different clades and hosts. P one. 2018:1-29.
10. Focosi D, Maggi F. Avian Influenza Virus A(H5Nx) and Prepandemic Candidate Vaccines: State of the Art. Molecular Sciences. 2024;25:1-16.
11. He J, Kam YW. Insights from Avian Influenza: A Review of Its Multifaceted Nature and Future Pandemic Preparedness. Viruses. 2024;16:1-14.
12. Faccin FC, Perez DR. Pandemic preparedness through vaccine development for avian influenza viruses. Human Vaccine & Immunotherapeutics. 2024;20(1):1-14.
13. Chinh CV, Quoc VP, Tan LH, Van DN, Quang TP, Van BL. Persistence of vaccine-induced antibodies to A/H5N1 at 30 months and 36 months after vaccination in Vietnam. Epi H. 2021;43:1-6.
14. Tan Y, He F, Kwang J. How can we develop universal H5N1 vaccines. Immunotherapy. 2015;1-4.
15. Simhadri VR, Dimitrova M, Mariano JL, Zenarruzabeitia O, Zhong W, Ozawa T, et al. A Human Anti-M2 Antibody Mediates Antibody-Dependent Cell-Mediated Cytotoxicity (ADCC) and Cytokine Secretion by Resting and Cytokine-Preactivated Natural Killer (NK) Cells. P one. 2015;1-13.
16. Gomes KB, Zhang YN, Lee YZ, Eldad M, Lim A, Ward G, et al. Single-Component Multilayered Self-Assembling Protein Nanoparticles Displaying Extracellular Domains of Matrix Protein 2 as a Pan-influenza A Vaccine. Acs nano. 2023;17:23545-67.
17. Liu TY, Hussein WM, Toth I, Skwarczynski M. Advances in Peptide-based Human Papillomavirus Therapeutic Vaccines. Current Topics in Medicinal Chemistry. 2012;12:1-12.
18. Coughlin MM, Beck AS, Bankamp B, Rota PA. Perspective on Global Measles Epidemiology and Control and the Role of Novel Vaccination Strategies. Viruses. 2017;9:1-13.
19. Vink P, Shiramoto M, Ogawa M, Eda M, Douha M, Heineman T, et al. Safety and immunogenicity of a Herpes Zoster subunit vaccine in Japanese population aged ≥50 years when administered subcutaneously vs. intramuscularly. Human Vaccine & Immunotherapeutics. 2017;13:3, 574-8.
20. Mytle N, Leyrer S, Inglefield JR, Harris AM, Hickey TE, Minang J, et al. Influenza Antigens NP and M2 Confer Cross Protection to BALB/c Mice against Lethal Challenge with H1N1, Pandemic H1N1 or H5N1 Influenza A Viruses. Viruses. 2021;13:1-13.
21. Kalaiyarasu S, Bhatia S, Mishra N, Kumar DS, Kumar M, Sood R, et al. Elicitation of Highly Pathogenic Avian Influenza H5N1 M2e and HA2-Specific Humoral and Cell-Mediated Immune Response in Chicken Following Immunization With Recombinant M2e–HA2 Fusion Protein. Frontiers in Veterinary Science. 2021 February 5;7:1-15.
22. Hendy DA, Amouzougan EA, Young IC, Bachelder EM, Ainslie KM. Nano/microparticle Formulations for Universal Influenza Vaccines. AAPS Journal. 2022;24:1-13.
23. Kostolansky F, Tomcikova K, Briestenska K, Mikusova M, Vareckova E. Universal anti-influenza vaccines based on viral HA2 and M2e antigens. Acta virologica. 2020;64:417-26.
24. Kim KH, Li Z, Bhatnagar N, Subbiah J, Park BR, Shin CH, et al. Universal protection against influenza viruses by multi-subtype neuraminidase and M2 ectodomain virus-like particle. PLOS Pathogens. 2022 August 25;1-23.
25. Subbiah J, Oh J, Kim KH, Shin CH, Park BR, Bhatnagar N, et al. A chimeric thermostable M2e and H3 stalk-based universal influenza A virus vaccine. NPG vaccine. 2022;7:68,1-15.