Vaccine Research
ISSN: 2423-4923
eISSN: 2383-2819

Immunogenicity Study of a Multi-Epitope Vaccine Prototype Against influenza A Virus in a Mice Model

Mina Mirzaee [1, 2], Fatemeh Fotouhi [2], Hadiseh Shokohi [2], Parisa Roshani Asl[2], Hadi Bamehr [2], Golnaz Bahramali [3], Behrokh Farhmand [2]*, Seyed Masoud Hosseini [1]*

[1]Department of Microbiology and Microbial Biotechnology, Faculty of Life Science and Biotechnology, University of Shahid Beheshti, Tehran, Iran. [2]Department of Influenza and Respiratory Viruses, Pasteur Institute of Iran, Tehran, Iran. [3]Department of Hepatitis and AIDS, Pasteur Institute of Iran, Tehran, Iran.

ARTICLE INFO

Research Article

VacRes, 2023
Vol. 10, No.2, 49 – 55
Received: July 06, 2025
Accepted: August 16, 2025
Pasteur Institute of Iran

***Corresponding Authors:**

Behrokh Farahmand;
Department of Influenza and Respiratory Viruses, Pasteur Institute of Iran, Tehran, Iran.
Email: b_farahmand@pasteur.ac.ir
Tel/Fax: (+98) 21 64112183/ (+98) 21 66496517

Seyed Masoud Hosseini;
Department of Microbiology and Microbial Biotechnology, Faculty of Life Science and Biotechnology, University of Shahid Beheshti. Tehran. Iran.
Email: ma_Hosseini@sbu.ac.ir
Tel/Fax: +98 21 29905913

KEYWORDS: Influenza virus, Multi-epitope, Peptide vaccine, Immunogenicity

ABSTRACT

Introduction: Influenza, particularly Influenza A, remains a significant global health challenge due to its high morbidity and mortality rates. The hemagglutinin (HA) protein plays a critical role in viral entry, replication, and disease progression, while neuraminidase (NA) is essential for the forming and releasing of viral particles. Despite the ongoing challenge of HA protein variability, which leads to the emergence of new antigenic strains, this study focuses on enhancing antibody responses and achieving partial protection against influenza through the use of HA and NA proteins. **Methods:** A recombinant peptide vaccine targeting Influenza A was developed and tested in mice, showing strong immune responses and increased protection. Evaluated through computational, laboratory, and animal studies, the vaccine proved effective both alone and with an adjuvant. Prior bioinformatics analyses ensured its safety and stability, highlighting the promise of using immunodominant epitopes in recombinant vaccines for better influenza prevention. **Results:** The recombinant peptide vaccine against Influenza A viruses demonstrated strong immune responses and enhanced protection in a mice model. Evaluations under *in silico*, *in vitro*, and *in vivo* conditions confirmed the vaccine's efficacy, both alone and with an adjuvant, through robust immune responses and cytokine production. These findings highlight the potential of bioinformatically guided immunodominant epitopes in recombinant vaccines for improved influenza control. **Conclusion:** This recombinant vaccine prototype shows strong immunogenicity and potential for effective influenza control in mice model.

INTRODUCTION

Effective vaccine development is fundamental in the prevention and control of infectious diseases, as it helps curb pathogen spread and mitigate adverse health outcomes[1]. In the context of influenza, the primary objective of vaccination is to induce a strong and protective immune response, thereby improving the body's defense against this rapidly mutating virus. Among the different influenza virus types, type A is the most readily transmissible to humans due to its wide range of subtypes. Both influenza A and B viruses possess genomes consisting of eight segments of negative-sense RNA[2]. Among the different types of influenza viruses, influenza A is the most transmissible to humans due to its wide variety of subtypes. The two major surface glycoproteins of the virus are hemagglutinin (HA) and neuraminidase (NA), which play key roles in viral entry and release[3]. Hemagglutinin (HA) and neuraminidase (NA) are the main antigenic proteins used to classify influenza A viruses into various subtypes. To date, sixteen antigenically distinct HA subtypes (H1–H16) and nine NA subtypes (N1–N9) have been identified. Additionally, a novel HA subtype, H17, has recently been discovered [4]. Currently, H1 and H3 virus lineages are prevalent in human circulation [5]. A key element in influenza vaccine development is the hemagglutinin (HA) protein, a vital surface antigen responsible for facilitating viral entry into host cells. Nonetheless, the high mutation rate of HA presents a major obstacle, enabling the virus to swiftly produce novel strains with altered antigenic properties, which may compromise the efficacy of current vaccines[6] .

[Downloaded from vacres.pasteur.ac.ir on 2026-09-15]

[DOI: 10.52547/vacres.10.2.49]

The HA glycoprotein is essential in the assembly of viral particles. By attaching to host cell receptors and promoting membrane fusion, it serves as a primary antigen that triggers the immune system to produce neutralizing antibodies[7]. Once the virus enters the host cell, hemagglutinin undergoes structural changes initiated by proteases like trypsin, which facilitate the fusion of viral and cellular membranes. Although genetic mutations occur, the HA2 subunit continues to serve as a key immune target because it contains more epitopes than HA1[8]. Besides hemagglutinin (HA), the neuraminidase (NA) protein of the influenza virus has garnered significant interest because of its involvement in viral replication and the progression of the disease. Nonetheless, efforts to develop vaccines specifically targeting NA remain limited. Consequently, designing a peptide-based vaccine that targets the immunodominant epitopes of both HA and NA proteins is essential for combating influenza type A[9].

Adjuvants enhance immune responses by activating both humoral and cellular immunity pathways. They play a crucial role, particularly for vulnerable populations who need rapid immune activation. Among these, aluminum salts -commonly referred to as alum- are the most extensively utilized adjuvants in vaccine formulations. The use of alum dates back to 1920 when Glenny and colleagues first combined it with the diphtheria vaccine[10, 11] . Advancements in epitope prediction algorithms and protein modeling techniques have significantly facilitated the design of three-dimensional vaccine constructs. The complex spatial arrangement of epitopes on influenza A surface proteins provides multiple promising targets for eliciting effective immune responses in mice models[12]. This paper aims to address these challenges by investigating novel strategies based on the design of a multi-epitope influenza vaccine using bioinformatics studies to enhance antibody responses. In particular, it focuses on HA and NA proteins as promising avenues for vaccine development.

MATERIALS AND METHODS

Our previous research focused on using bioinformatics approaches to design a novel vaccine. This vaccine contains a combination of key protein fragments derived from the hemagglutinin and neuraminidase proteins of the influenza virus. These fragments were selected for their ability to elicit immune responses and for being conserved.

Ethics Statement

All animal procedures were approved by the Ethics Committee of the Pasteur Institute of Iran (IR.PII.REC.1400.064) and were carried out according to their established guidelines. BALB/c mice (Pasteur Institute of Iran, Tehran, Iran, Female ,6-8 weeks old) were kept in a room with adequate lighting (12-h light/dark cycle) and temperature-controlled conditions (26 ± 1°C) with a humidity level of 50 ± 10%. They were allowed free access to a standard diet and water.

Recombinant Protein Construction

To develop the recombinant protein, three Influenza A virus strains—H1N1 and H3N2—were chosen for analysis. Both major surface proteins, Hemagglutinin (HA) and Neuraminidase (NA), from each strain were examined. Protein sequences were obtained from the UniProt database (https://www.uniprot.org). Subsequently, B-cell and T-cell epitopes were predicted via the IEDB server (https://www.iedb.org). From these predictions, epitopes demonstrating high conservation, immunodominance, and antigenicity were selected for further study. The chosen epitopes were connected using specific linkers to maintain their functionality: B-cell epitopes were linked with the flexible GPGPG sequence, while T-cell epitopes were joined using the rigid EAAAK linker. Additionally, a triple GPGPG linker was incorporated between the B-cell and T-cell epitopes to preserve both structural integrity and functional separation. Three-dimensional modeling of the recombinant protein was conducted using the I-TASSER server. Following this, molecular dynamics simulations were performed to assess the construct's structural stability. Key parameters analyzed included RMSD, RMSF, radius of gyration (Rg), and hydrogen bond interactions. Moreover, molecular docking results indicated that the vaccine candidate can effectively engage immune receptors such as TLR3, TLR7, and TLR8, which are vital for mounting a protective response against influenza. Finally, the recombinant protein, consisting of 199 amino acids, was synthesized and cloned into the pET21a vector by ShineGene Biotech Company, The BamHI restriction site was introduced at the beginning of the sequence (5/-end), and the XhoI site was added at the end (3/-end), and subsequent steps were performed according to the following procedures[13].

Recombinant Protein Expression

The recombinant vector was successfully expressed in *Escherichia coli* BL21 strain, and protein expression was validated using SDS-PAGE and Western blot analysis. Briefly, competent *E. coli* cells were transformed with the recombinant plasmid and spread onto LB agar plates containing kanamycin (50 µg/ml) and tetracycline (10 µg/ml). The plates were incubated at 37°C for 16 h. Single colonies were then inoculated into 10 ml LB broth supplemented with the same antibiotics and cultured for 2–3 h at 37°C with shaking at 185 rpm. Protein expression was induced by adding IPTG to a final concentration of 0.5 mM, followed by incubation at 37°C with shaking at 195 rpm. For large-scale production, LB broth was inoculated with a single recombinant colony, and after cultivation, bacterial cultures were harvested by centrifugation at 10,000 rpm for 10 min at 4°C. The resulting cell pellets were subsequently stored at –20°C for further use.

Extraction of Recombinant Protein

Protein extraction was carried out utilizing LEW buffer combined with 8 M urea. A solution comprising 50 mM monobasic sodium phosphate (NaH_2PO_4) and 300 mM sodium chloride (NaCl) in LEW buffer was mixed with the pellet at a weight ratio of 1:5 (W/W). The suspension was homogenized by gentle pipetting, incubated at 4 °C for 30 min, and subsequently subjected to sonication under conditions of 25–30 pulses at 80–90% pulse amplitude with 30 s on/off cycles. After sonication, the samples were centrifuged at 10,000 rpm at 4 °C for 5 mins. Both the supernatant and pellet fractions were then analyzed by SDS-PAGE.

Protein Purification using Ni-NTA Chromatography Column

Based on the manufacturer's instructions, the recombinant protein underwent further purification through Ni-NTA affinity chromatography (QIAGEN, Germany). Elution was carried out using imidazole (Merck, Germany) buffers at concentrations of 20, 30, 40, 150, and 250 mM, each containing 8 M urea (Behansar, Iran). The purified vaccine candidate proteins were subsequently assessed by SDS-PAGE analysis.

[Downloaded from vacres.pasteur.ac.ir on 2026-09-15]

[DOI: 10.52547/vacres.10.2.49]

Virus Inoculation for Influenza Vaccine Development

MDCK cells were seeded at a density of 2×10^5 cells per well and cultured overnight until reaching approximately 80% confluency. Prior to transfection, cells were transferred directly into culture flasks containing DMEM (Sigma Aldrich company, USA) supplemented with 20% serum, 100 U/ml penicillin, and 100 µg/ml streptomycin. After 4 h, the culture medium was replaced to eliminate the cytotoxic effects of DMSO. Following multiple passages and adaptation to laboratory conditions, cells were prepared for inoculation. Optimal inoculation was achieved when approximately 80% of the flask's adhesive surface was covered by cells. Before virus inoculation, cells were washed with 3 ml of PBS to remove residual serum, which can inhibit trypsin activity necessary for HA0 cleavage. A minimal volume of virus dilution sufficient to cover the cell surface was then added and incubated at 37°C for one hour to allow viral adsorption. Subsequently, serum-free DMEM containing TPCK-treated trypsin (Merck, Germany) was added. Cultures were maintained in a CO_2 incubator at 37°C and monitored for cytopathic effects (CPE) over two days. Virus titers were quantified using hemagglutination assay and the $TCID_{50}$ method.

Animal Immunization Protocol

To evaluate the immunogenicity of the chimeric protein, BALB/c mice were used in the study. The animals were randomly divided into two experimental groups, each consisting of 17 mice, and three control groups, which included 22 mice in the PBS group and 9 mice in the inactivated virus groups. The experimental groups received the recombinant protein either alone or in combination with the Alum adjuvant (Brenntag Biosector, Denmark (CAS Number:21645-51-2)) . The control groups received PBS (negative control) or inactivated strains of H1N1 and H3N2 viruses (positive control).

The injections were performed subcutaneously in a total volume of 100 µl, containing 15 µg of the chimeric protein, and were given three times over a 21-day period (as shown in Fig. 1 and Table 1).

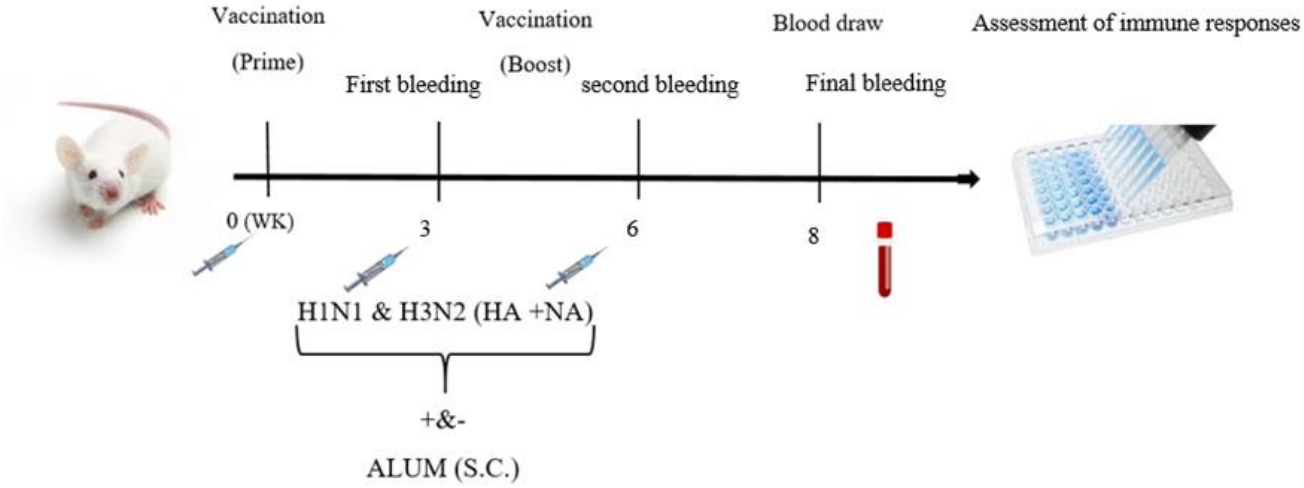

Fig. 1. Schematic illustration of mice Immunization. Mice were immunized subcutaneously (S.C.) at weeks 0 (prime) 3 and 6 (boost) with a combination of H1N1(A/H1N1/Tehran) and H3N2(A/H3N2/X47) influenza antigens (HA + NA), with or without Alum as an adjuvant. Blood sampling was performed after each vaccine injection for the assessment of immune responses.

Table 1. Immunization of experimental animal groups. The compounds were administered subcutaneously in a total volume of 100 µl containing 15 µg of the recombinant protein, either alone or with adjuvant.

Number	Animals per group	Compound injection (100 µl) (three times with 21-day interval)
1	17	15 µg Recombinant protein (AHN)
2	17	15 µg Recombinant protein + Alum (AHN+Alum)
3	11	PBS
4	9	Inactivated virus A(H1N1)
5	9	Inactivated virus A(H3N2)

Assessment of Antibodies Specific to the Recombinant Protein

Two weeks following the final immunization, blood samples were collected from 4 mice per group to evaluate immune responses, including total IgG as well as IgG1 and IgG2a subclasses. The assessment was conducted using the ELISA method. In brief, 96-well plates were coated overnight at 4°C with 100 µl of recombinant protein at a concentration of 2.5 mg/ml. Mouse sera were diluted 1:500 in PBS, with optimal antigen coating and serum dilutions determined through checkerboard titration. Washing steps were performed using PBS (10 mM, pH 7.4) supplemented with 0.05% Tween-20 (PBS-T). Blocking was carried out using PBS-T containing 5% bovine serum albumin to minimize nonspecific binding. To detect total IgG, an HRP-conjugated goat anti-mouse IgG antibody (Sigma-Aldrich company, USA) was used as the secondary antibody, and absorbance was measured at 450 nm. For IgG subclass analysis, goat anti-mouse IgG1 and IgG2a antibodies served as secondary antibodies, followed by a tertiary incubation with HRP-conjugated rabbit anti-goat IgG antibody (Sigma-Aldrich company, USA)[14].

[Downloaded from vacres.pasteur.ac.ir on 2026-09-15]

[DOI: 10.52547/vacres.10.2.49]

Cytokine Assay

Two weeks following the final immunization, spleens were aseptically harvested from three mice per group to assess cellular immune responses. Mononuclear cells were isolated, and lymphocytes were cultured in vitro in the presence of specific antigens. Briefly, splenocytes were seeded into 96-well plates and incubated at 37°C with 5% CO_2, either stimulated with 1 µg/mL recombinant protein or left unstimulated as controls. After 72 h, culture supernatants were collected. According to the manufacturer's protocol, levels of secreted IFN-γ and IL-6 were quantified using sandwich ELISA kits (R&D Systems, DuoSet ELISA, USA). Optical densities were measured at 450 nm, and cytokine concentrations were calculated based on standard curves.

VNT-Test

Neutralizing antibody titers were determined in a VNT test. Briefly, MDCK cells were seeded and grown in 96-well plates 24 h before infection. Serum samples were serially diluted (2-fold dilution) in DMEM then mixed with 100 $TCID_{50}$/mL of H1N1 and H3N2. After incubation for 2 h, the serum-virus mixture was transferred onto MDCK at 37°C and 5% CO_2. Finally incubated with DMEM containing 1 µg/mL TPCK-treated trypsin for 72 h. Neutralization was evaluated by HA test and light microscopy for the absence of specific CPE.

Statistical Analysis

To analyze the immune response assays, the findings were displayed using a one-way ANOVA. *P* values less than 0.01 were considered statistically significant. The program GraphPad Prism 9 (Graphpad software, Inc ,USA) was used to analyze the data.

RESULTS

Bioinformatics Evaluation of Recombinant Influenza Vaccine Design

Our research used bioinformatics methods to design a novel vaccine consisting of key fragments from the hemagglutinin (HA) and neuraminidase (NA) proteins of the Influenza A virus. B-cell and T-cell epitopes were predicted and optimally linked together. The 3D modeling and molecular dynamics simulations showed that this vaccine can potentially activate the immune system via TLR3, TLR7, and TLR8 receptors. Molecular docking revealed strong binding affinities, particularly with TLR3 (ΔG = –21.5 kcal/mol, 9 hydrogen bonds, interface area: 1808 Å², docking score: –308.34), followed by TLR7 (ΔG = –10.9 kcal/mol, 8 hydrogen bonds, 994 Å², docking score: –300.24), and TLR8 (ΔG = –11.6 kcal/mol, 7 hydrogen bonds, 1397 Å², docking score: –297.33). The recombinant protein was cloned into the pET21a vector and confirmed in the Materials and Methods section. The *in silico* results indicated that this vaccine has a high potential to combat a wide range of Influenza viruses.

Production of Recombinant Protein

The presence of the cloned recombinant vector containing conserved regions of the influenza virus protein was verified by enzymatic digestion and sequencing (data not shown). Subsequently, large-scale expression of the recombinant protein was performed in *E. coli* BL21 cells. The protein was purified using Ni-NTA affinity chromatography and then dialyzed against PBS to remove residual urea and imidazole. Successful expression of the recombinant protein was confirmed by SDS-PAGE and Western blot analysis, revealing a protein band corresponding to approximately 21 kDa (Fig. 2)[13].

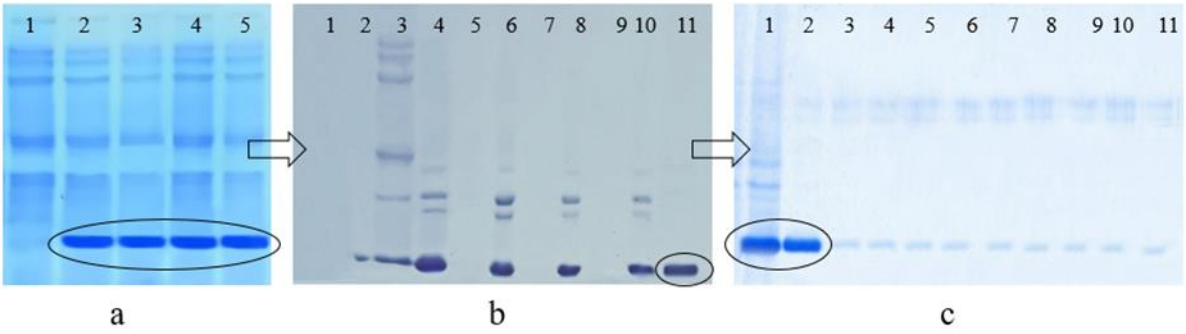

Fig. 2. Steps of expression, extraction, and purification of the recombinant protein.**(a)** SDS-PAGE result of protein expression; lane1 before induction, lane 2-5, 1to 4 h after induction using IPTG (0.5mM).**(b)** Electrophoresis results related to the extraction of recombinant protein from *E. coli* strain BL21 lanes (1,3,5,7,9 and 11) are supernatant and lanes (2,4,6,8,10) are pellets' wells .**(c)** SDS-PAGE result of protein purification using imidazole(20-250mM), lane(1-2); imidazole 20mM; lanes (3-4) imidazole 30mM; and lane (5-6); imidazole 40mM , lanes(7-8) imidazole 150mM, lane (9-11); imidazole 250mM .

Specific Antibodies Measurement

The levels of specific antibodies against the AHN protein were evaluated in a mice model. The animals were immunized according to the experimental protocol. Blood samples were collected two weeks after each immunization and analyzed using the ELISA assay. The results showed that antibody titers significantly increased after the third injection compared to the first and second immunizations for the adjuvanted vaccine candidate (Fig. 3a). An independent experiment was conducted among all treatment and control groups, and a statistically significant difference was observed in the AHN with Alum group (Fig. 3b). Additionally, AHN alone was able to induce both IgG1 and IgG2a antibodies, indicating a mixed Th1/Th2 immune response. Specific antibody responses to AHN were measured and compared with control groups. As expected, the AHN + Alum group exhibited significantly higher specific IgG titers than the AHN-alone and control groups ($P < 0.0001$; Fig. 3c).

[Downloaded from vacres.pasteur.ac.ir on 2026-09-15]

[DOI: 10.52547/vacres.10.2.49]

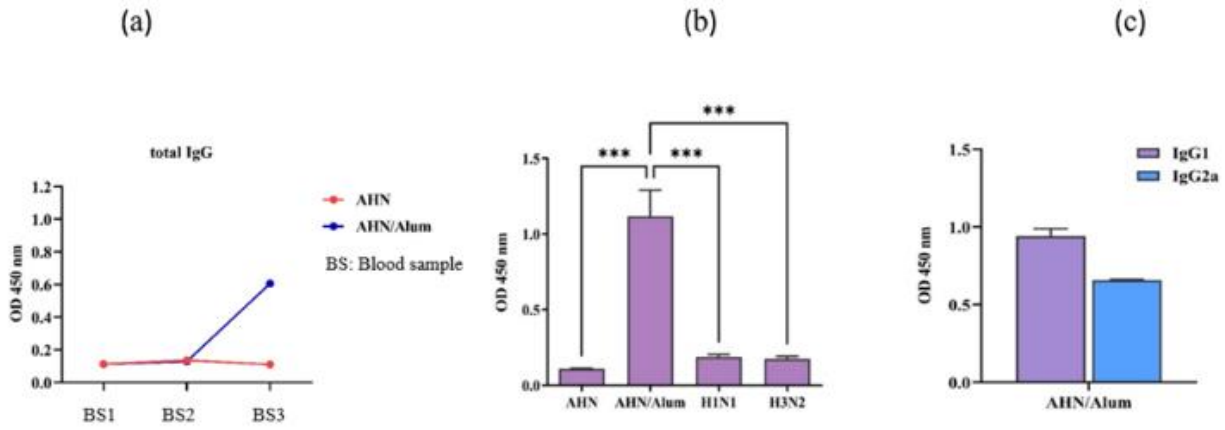

Fig. 3. Assessment of IgG antibodies in the sera of the vaccinated mice. (a) IgG antibodies in vaccinated mice's serum were measured using the ELISA method: Individual serum values at 450 nm OD were compared across groups receiving three doses with and without adjuvant. (b) Across 4 independent experiments, ANOVA results (P = 0.0001) indicated statistically significant differences between all treatment and control groups. The figure denotes additional differences as **P* < 0.05, ***P* < 0.01, ****P* < 0.001. (c) IgG1 and IgG2a antibodies in vaccinated mice's serum were measured using the ELISA method: Individual serum values at 450 nm OD were recorded.

Cytokine Assay Results

To evaluate the potency of the chimeric protein in inducing an immune response, the secretions of IFN-γ as a key Th1 cytokine and IL-6 as a major cytokine involved in inflammatory and humoral immune responses were assessed in splenocytes of the immunized mice. For this purpose, spleen cells were harvested 10 days after the final immunization, stimulated with the specific antigen, and the concentrations of IFN-γ and IL-6 in the culture supernatant were subsequently measured. The results shown in Fig. 4 indicate that both immunized groups had higher cytokine production, compared to the negative control groups. Importantly, the group that received AHN- only showed a significant increase in both IFN-γ and IL-6 production compared to the AHN+Alum (*P* < 0.001; Fig 4).

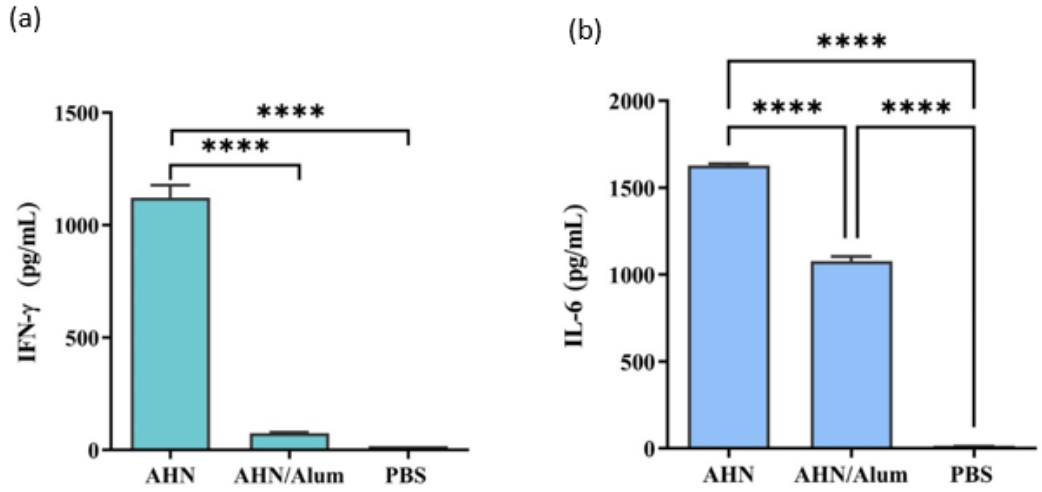

Fig. 3. Assessment of IgG antibodies in the sera of the vaccinated mice. (a) IgG antibodies in vaccinated mice's serum were measured using the ELISA method: Individual serum values at 450 nm OD were compared across groups receiving three doses with and without adjuvant. (b) Across 4 independent experiments, ANOVA results (P = 0.0001) indicated statistically significant differences between all treatment and control groups. The figure denotes additional differences as **P* < 0.05, ***P* < 0.01, ****P* < 0.001. (c) IgG1 and IgG2a antibodies in vaccinated mice's serum were measured using the ELISA method: Individual serum values at 450 nm OD were recorded.

VNT Test Results

Neutralization assay results indicated that the antibodies elicited by vaccination were capable of neutralizing influenza virus A/H3N2 (100 $TCID_{50}$) at a serum dilution of 1:8, whereas a lower neutralizing activity was observed against influenza virus A/H1N1, with effective neutralization at a 1:2 dilution.

[Downloaded from vacres.pasteur.ac.ir on 2026-09-15]

[DOI: 10.52547/vacres.10.2.49]

Tabel 2. Serum neutralization titers against influenza A/H1N1 and A/H3N2 viruses in different experimental groups. The numbers indicate the highest serum dilution at which effective virus neutralization was observed. The "–" symbol denotes no detectable neutralizing activity.

	AHN group	AHN/Alum group	H1N1 group	H3N2 group	PBS group
H1N1	-	1:2	1:16		-
H3N2	1:8	1:8		1:16	-

DISCUSSION

Each year, respiratory infections such as influenza account for a significant proportion of acute illnesses and mortality worldwide, posing the greatest risk to older adults, young children, and people with pre-existing medical conditions[14]. The high mutation rate and genetic diversity of influenza viruses pose significant obstacles to current prevention methods, highlighting the need for continuous improvement and diversification of vaccine platforms to achieve broader and longer-lasting protection. Conventional egg-based vaccines are expensive, slow to produce, and may cause allergic responses, whereas live attenuated vaccines can result in side effects, particularly among individuals with weakened immune systems[15, 16]. Developing recombinant protein vaccines using advanced biotechnological methods is crucial for overcoming these challenges. Emphasis on conserved viral proteins is key to ensuring the efficacy and longevity of influenza vaccines.

To achieve optimal efficacy, a vaccine must stimulate comprehensive immune activation, ensuring the development of lasting immunity against the target pathogen. This includes the coordinated engagement of both innate and adaptive immune responses—particularly the activation of Th1 and Th2 pathways alongside the induction of robust humoral immunity[16].

In the present study, a recombinant protein derived from influenza virus strains A (H1N1/Tehran) and A(H3N2/X47) was successfully expressed and purified using a prokaryotic expression system. To evaluate the immunogenic potential of the vaccine candidates, both humoral and cellular immune responses were examined. The humoral response was assessed by quantifying total IgG, IgG1 subclasses, and performing the virus neutralization test (VNT). Cellular immunity was evaluated through measurement of IgG2a and IFN-γ, while the inflammatory profile was characterized by determining IL-6 levels[17]. The anti-AHN antibodies in mice that received AHN with alum primarily comprised the IgG1a subclass, indicating the humoral immunity activity index.

In 2003, HUA and et al. showed that by using 3 linear HA epitopes that cause Strong humoral immunity was induced in mice and rabbits, which enhanced the immunogenicity of the epitopes and elicited a strong neutralizing antibody response[18]. In this study, it was shown that the recombinant multi-epitope vaccine can induce antiviral activities against influenza virus, which may provide a new way to produce an effective vaccine against this influenza A virus. These results were consistent with our results. Also in 2020, Giurgea et al. stated that NA-based immunity may have the ability to overcome many of the deficiencies associated with current vaccines[19]. This study specifically emphasized the significance of immunodominant and conserved epitopes of neuraminidase, resulting in favorable outcomes. As a conclusion, since the NA content in existing inactivated influenza vaccines is not low, changing production practices to optimize and standardize NA content may be a quick way to improve existing vaccine strategies because it is likely that safety caused by NA is an important option for future influenza vaccine strategy[20].

In 2014, Jacob et al. developed the M-001 universal influenza vaccine. It contains conserved epitopes of influenza A, capable of triggering both cellular and humoral immune responses, providing protection against various strains of influenza A and B[21]. Also, in 2024, Lifang Yuan and her colleagues designed a multi-epitope vaccine (MEV) against influenza that The immunological simulations showed that could induce high levels of both B-cell and T-cell immune responses[22]. Several studies indicate that solely inducing a humoral immune response is insufficient for full protection against Influenza A infection, providing only partial protection[23]. Developing a comprehensive vaccine requires stimulation of both humoral and cellular responses. In this study, the levels of humoral and cellular immune stimulations were examined by measuring IgG1 and IgG2a subtype antibodies and the results indicated that The IgG1/IgG2a ratio indicates that this ratio is slightly above 1, suggesting a tendency toward a humoral immune response.

Evidence suggests IL-6 triggers a series of signaling events primarily linked to the JAK/STAT3 activation pathway [24-26], leading to the transcription of numerous downstream genes associated with signaling mechanisms. Cell signaling encompasses cytokines, receptors, adapter proteins, and protein kinases. Additionally, it regulates the production of proteins involved in gene expression regulation. The multitude of genes influenced by IL-6 activity may account for the diverse nature of this interleukin, resulting in both pro-inflammatory and anti-inflammatory effects[27]. The levels of IL-6 are less stable than other inflammatory markers like CRP[28]. The expression of this cytokine by the vaccine candidate can be valuable. As it can be seen in the results, AHN significantly increased the induction of this cytokine, compared to PBS and even compared to AHN/ALUM.

Despite the widespread use of aluminum in pharmaceuticals and vaccines, the minimum effective immunogen dose and its application in humans are constrained by potential neurotoxic effects. The results of this investigation underscored a robust cellular immune response to the recombinant protein without adjuvant, suggesting a promising avenue for potentially omitting the adjuvant and emphasizing the advantages of this particular recombinant protein. Both IFN-γ and IL-6 productions were demonstrated to be increased in mice, following AHN vaccination, signifying effective activation of the immune response and readiness to combat the viruses. In 2024, Lifang Yuan and his colleagues conducted a study on mice that showed IFN-γ as an indicator of cellular immune system stimulation and immunoglobulin G and HI and MN antibodies as an indicator of humoral immune system stimulation. Their MEV (multi epitope

[Downloaded from vacres.pasteur.ac.ir on 2026-09-15]

[DOI: 10.52547/vacres.10.2.49]

vaccine) demonstrated the efficacy of a broad-spectrum against influenza viruses in mouse [29].

The present investigation underscored a robust cellular and humoral immune response to the recombinant protein with or without adjuvant. The results of the neutralization assay showed that the antibodies elicited by vaccination were capable of neutralizing influenza virus A/H3N2 (100 $TCID_{50}$) at a serum dilution of 1:8, whereas a lower neutralizing activity was observed against influenza virus A/H1N1, with effective neutralization at a 1:2 dilution. These results clearly demonstrate that the developed vaccine induces antibodies with the ability to neutralize different influenza viruses, although the response is stronger against some strains like A/H3N2 compared to others, such as A/H1N1. This may be due to antigenic differences and the characteristics of the viruses, highlighting the need for optimization of vaccines to enhance its efficacy against a broader range of strains.

In conclusion, the development of effective and safe vaccines against influenza virus type A is of paramount importance. In this project, were designed and constructed a peptide vaccine comprising peptides targeting immunodominant epitopes of hemagglutinin and neuraminidase. The experiments showed that the peptide vaccine could induce robust antibody responses in a mouse model and enhance protection against influenza. These findings suggest that the use of peptides containing immunodominant epitopes in influenza vaccines can be an effective approach for disease control and prevention. Although the findings of this study are preliminary, they provide valuable insights into the potential of the vaccine candidate. Further comprehensive investigations in different experimental models and eventually in clinical settings are necessary to fully evaluate its safety and efficacy.

ACKNOWLEDGEMENTS

This study was conducted at the Department of Influenza and other Respiratory Viruses, Pasteur Institute of Iran, Tehran (Grant Number 1262). We extend our gratitude to all colleagues for their invaluable assistance.

CONFLICT OF INTEREST

The authors declare they have no conflict of interests.

REFERENCES

1. Rodrigues CM, Plotkin SA. Impact of vaccines; health, economic and social perspectives. Frontiers in microbiology. 2020;11:1526.
2. Lopez CE, Legge KL. Influenza A virus vaccination: immunity, protection, and recent advances toward a universal vaccine. Vaccines. 2020;8(3):434.
3. Bouvier NM, Palese P. The biology of influenza viruses. Vaccine. 2008;26:D49-D53.
4. Das K, Aramini JM, Ma L-C, Krug RM, Arnold E. Structures of influenza A proteins and insights into antiviral drug targets. Nature structural & molecular biology. 2010;17(5):530-8.
5. Odun-Ayo F, Odaibo G, Olaleye D. Influenza virus A (H1 and H3) and B co-circulation among patient presenting with acute respiratory tract infection in Ibadan, Nigeria. African health sciences. 2018;18(4):1134-43.
6. Lim CML, Komarasamy TV, Adnan NAAB, Radhakrishnan AK, Balasubramaniam VR. Recent advances, approaches and challenges in the development of universal influenza vaccines. Influenza and Other Respiratory Viruses. 2024;18(3):e13276.
7. Sriwilaijaroen N, Suzuki Y. Molecular basis of the structure and function of H1 hemagglutinin of influenza virus. Proceedings of the Japan Academy, Series B. 2012;88(6):226-49.
8. Russell CJ. Acid-induced membrane fusion by the hemagglutinin protein and its role in influenza virus biology. Influenza Pathogenesis and Control-Volume I. 2014:93-116.
9. Vogel OA, Manicassamy B. Broadly protective strategies against influenza viruses: universal vaccines and therapeutics. Frontiers in microbiology. 2020;11:135.
10. Shi ShuTing SS, Zhu HaoRu ZH, Xia XinYu XX, Liang ZhiHui LZ, Ma XueHu MX, Sun BingBing SB. Vaccine adjuvants: understanding the structure and mechanism of adjuvanticity. 2019.
11. Di Pasquale A, Preiss S, Tavares Da Silva F, Garçon N. Vaccine adjuvants: from 1920 to 2015 and beyond. Vaccines. 2015;3(2):320-43.
12. Mubarak AS, Ameen ZS, Hassan AS, Ozsahin DU. Enhancing tuberculosis vaccine development: a deconvolution neural network approach for multi-epitope prediction. Scientific Reports. 2024;14(1):10375.
13. Mirzaee M, Hosseini SM, Farahmand B, Fotouhi F, Bahramali G. A novel multi–epitope-based peptide recombinant influenza A vaccine prototype utilizing neuraminidase and hemagglutinin surface proteins: From in silico to preliminary study. Computational Biology and Chemistry. 2025;117:108411.
14. Saleh M, Nowroozi J, Farahmand B, Fotouhi F. An approach to the influenza chimeric subunit vaccine (3M2e-HA2-NP) provides efficient protection against lethal virus challenge. Biotechnology letters. 2020;42:1147-59.
15. Adar Y, Singer Y, Levi R, Tzehoval E, Perk S, Banet-Noach C et al. A universal epitope-based influenza vaccine and its efficacy against H5N1. Vaccine. 2009;27(15):2099-107.
16. Ben-Yedidia T, Arnon R. Towards an epitope-based human vaccine for influenza. Human vaccines. 2005;1(3):95-101.
17. Liu C, Chu D, Kalantar-Zadeh K, George J, Young HA, Liu G. Cytokines: from clinical significance to quantification. Advanced Science. 2021;8(15):2004433.
18. Li H, Ding J, Chen Y-H. Recombinant protein comprising multi-neutralizing epitopes induced high titer of antibodies against influenza A virus. Immunobiology. 2003;207(5):305-13.
19. Giurgea LT, Morens DM, Taubenberger JK, Memoli MJ. Influenza neuraminidase: a neglected protein and its potential for a better influenza vaccine. Vaccines. 2020;8(3):409.
20. Stadlbauer D, Zhu X, McMahon M, Turner JS, Wohlbold TJ, Schmitz AJ et al. Broadly protective human antibodies that target the active site of influenza virus neuraminidase. Science. 2019;366(6464):499-504.
21. Atsmon J, Caraco Y, Ziv-Sefer S, Shaikevich D, Abramov E, Volokhov I et al. Priming by a novel universal influenza vaccine (Multimeric-001)—A gateway for improving immune response in the elderly population. Vaccine. 2014;32(44):5816-23.
22. Yuan L, Li X, Li M, Bi R, Li Y, Song J et al. In silico design of a broad-spectrum multiepitope vaccine against influenza virus. International Journal of Biological Macromolecules. 2024;254:128071.
23. Hauge S, Madhun A, Cox R, Brokstad K, Haaheim L. A comparison of the humoral and cellular immune responses at different immunological sites after split influenza virus vaccination of mice. Scandinavian journal of immunology. 2007;65(1):14-21.
24. Tanaka T, Narazaki M, Kishimoto T. IL-6 in inflammation, immunity, and disease. Cold Spring Harbor perspectives in biology. 2014;6(10):a016295.
25. Aliyu M, Zohora FT, Anka AU, Ali K, Maleknia S, Saffarioun M et al. Interleukin-6 cytokine: An overview of the immune regulation, immune dysregulation, and therapeutic approach. International immunopharmacology. 2022;111:109130.
26. Johnson DE, O'Keefe RA, Grandis JR. Targeting the IL-6/JAK/STAT3 signalling axis in cancer. Nature reviews Clinical oncology. 2018;15(4):234-48.
27. Al-Qahtani AA, Alhamlan FS, Al-Qahtani AA. Pro-inflammatory and anti-inflammatory interleukins in infectious diseases: A comprehensive review. Tropical medicine and infectious disease. 2024;9(1):13.
28. Radin AS, Kuhlman KR, Boyle CC, Haydon MD, Bower JE. Using the influenza vaccine as a mild, exogenous inflammatory challenge: When does inflammation peak? Brain, Behavior, & Immunity-Health. 2021;13:100239.
29. Yuan L, Zhang S, Bi R, Liu X, Han Z, Li M et al. A broad-spectrum multiepitope vaccine against seasonal influenza A and B viruses in mice. Ebiomedicine. 2024;106.

[Downloaded from vacres.pasteur.ac.ir on 2026-09-15]

[DOI: 10.52547/vacres.10.2.49]