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Evaluating the Immune Response of Recombinant H1N1 Hemagglutinin with MF59 Adjuvant in Animal Model as a Novel Alternative to the Influenza Vaccine

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ABSTRACT

The H1N1 influenza virus is known as a serious pandemic threat across the globe. Vaccination is one of the most effective methods of protection against this virus and the way to reduce the seasonal pandemic risk. The commercial vaccine does not adequately respond to pandemic strains. This study examines the potential function of formulated H1N1 hemagglutinin with MF59 adjuvant against A/PR/8/34 (H1N1). To this end, a recombinant hemagglutinin (rHA) gene of influenza A virus was designed and expressed in SF9 cell by the Baculovirus expression system.

Four groups of mice were immunized by rHA in combination with MF59, Alum adjuvant, and virus split only. The immunized mice subsequently used for the humoral immune assay and the results compared with untreated mice (negative group). Besides, both treated and control mice groups were challenged with mouse-adapted influenza virus A/PR/8/34(H1N1) through the intranasal drop. Bodyweight, survival, temperature variation, and the medical conditions of the samples were assessed. Mice immunized with the recombinant protein demonstrated a humoral response to the influenza A virus.

Upon virus challenging, co-administration of rHA with MF59 adjuvant could lead to 92% survival of the vaccinated mice within 10 days. The MF59-treated group showed slight weight loss and high-temperature body two weeks after infection. This group also displayed a higher hemagglutination inhibition (HI) antibody titer as compared to the group vaccinated with virus split, and Alum adjuvant.

Altogether, the results showed that the recombinant protein with the MF59 adjuvant created better safety than the Alum adjuvant, thereby can be considered as a safe and reliable vaccine against the H1N1 virus for further investigations.

Keywords: Baculoviruses; Hemagglutinin; Humoral immunity; H1N1 virus; Sf9 cells

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INTRODUCTION

Influenza is a zoonotic disease caused by RNA viruses from the *Orthomyxoviridae* family and has adversely influenced poultry and mammals.[1] About ten percent of the world's population suffers from influenza annually. Influenza strains cause a variety of symptoms depending on the host's immune system condition, ranging from the common cold to very severe complications and even death; additionally, they have the potential to damage throughout the respiratory tract.[2] Over time, each pandemic resulted from a novel strain of the virus, which threatened the health of millions of people all around the globe. For example, 50 million, one million, and 20 million people died by pandemics in Spain (1919-1918), Asia (1957) and Hong Kong (1968), respectively.[3,4] It is well-established that the viruses causing such pandemics are mainly emerging by transmission from other animal species and birds to human beings.[5,6] In April 2009, a new strain of influenza virus, which comprised a mixture of avian, human, and pig influenza viral genes, was recorded for the first time. This strain dubbed the swine influenza (H1N1/A), and primary appeared in Mexico, the United States and several other countries, and the World Health Organization (WHO) officially declared a pandemic on June 11, 2009.[7]

The triple influenza vaccine (TIV) is the most common human vaccine, which contains inactive and purified material from three viral strains comprising two types of influenza A and one type B.[8,9] However, TIV has no risk of transmitting the disease and has a poor activity. As the influenza virus is rapidly changing; therefore, it is highly recommended to provide a novel vaccine against the virus, instated of administration of the same vaccine for years.[8,11] Systematically, influenza viruses are RNA-based which include three of the seven genera of Orthomyxoviridae family, Influenza A, Influenza B, and Influenza C.[12] Moreover, their genome is not a single piece of nucleic acid; instead, it contains seven or eight pieces of segmented negative-sense RNA ones that encode the hemagglutinin (HA).[13]

The HA of influenza A virus plays an essential role as the major surface glycoprotein in mediating the entry and attachment of the virus to the host cells.[2] The epidemic diseases have occurred based on the alterations in the antigenic structure, therefore the HA could be the most effective target for the vaccination industry.[14]

Antibodies in the serum play a key role in the resistance to the infection as well as the cure for influenza. The effect of antibodies on protection against influenza virus disease depends on various factors such as age, infection, and antibody levels induced during previous infections.[15] During influenza virus infection, antibodies against HA, neuraminidase (NA), nucleoprotein (NP), and matrix proteins produced.[2]Induction of the formation of antibodies against the influenza virus surface antigens is a typical mechanism of modern influenza vaccines.[5] The requirement of annual renewal of the strain composition of vaccines is inevitable as the influenza virus surface proteins undergo progressive antigenic variation.[16] Influenza vaccines are often prescribed for high-risk groups, such as children and the elderly or those with diabetes, heart disease, or immunocompromised people.[17,18]

Adjuvant helps to build up the immune system mechanisms.[19] MF59 is oil in water-based adjuvant and has been used to stimulate cellular and humoral immune responses in the influenza vaccine.[20] Since 1983, Baculoviruses introduced as the gene carrier. This system has been recognized as one of the most powerful in eukaryotic protein expression systems. To date, a wide range of viral, fungal, plant, and animal genes have been expressed in insect cells using this method.[21,22] The recombinant proteins produced in the insect's Baculovirus expression system form 1 to 50 percent of all proteins in the cell.[21] It is noteworthy that the post-translational processes including glycosylation, phosphorylation, polymerization, and acetylation as well as protein folding on produced recombinant proteins are well performed by this system.[23] In addition, the cytoplasmic conditions of the insect cells provide the necessary position for the formation of disulfide bonds. Thus, the produced protein retains its original structure and can act similarly to natural protein and the glycosylation of HA occur very close to the human body. So we can expect more stable antibody production and more sustainable immunity using this expression system.[21,23] This study aimed to express the recombinant hemagglutinin (rHA) protein in sf9 insect cells by Bac to Bac expression system that it has been formulated with the MF59 adjuvant to enhance immune-accessibility and induction of protective immune systems against the H1N1 virus in mice.

MATERIALS AND METHODS

H1N1 HA Expression Vector Design and Construction

The full-length cDNA of influenza virus strain A/PR8/34(H1N1) was synthesized by RT-PCR through general primers, U12 and U13. Then, the *HA* gene was amplified by specific primers (Table 1), which were designed by Gene Runnerversion 3.05 and Oligo 4.0 (National Biosciences, Inc., Plymouth, MN) software. The gene of interest was inserted into commercial pFastBac HT A vector and transformed into *E. coli* TOP10 strain according to the method described by Sambrook and Russell 2001.[24] The vector contains a promoter of the *Baculovirus* polyhedrin gene, N-terminal 6xHis tag, and TEV protease cleavage site for removal of tag tail through purification. The plasmid was isolated by Qiagen kit (Qiagen GmbH, Germany) according to the instruction of the kit. Then confirmed using PCR assay and double digestion with *BamH*I and *Xho*I (Jena Bioscience, Germany), and loaded on 1% agarose gel. Subsequently, the correct pFastBac HT A vector transformed into DH10Bac to create recombinant Bacmid.[25] The Bacmid (trade name bMON14272) contains a kanamycin resistance marker. A mini-F replicon and a fragment of DNA encodes the LacZα peptide as well as a site for binding the bacterial transposon (mini att-Tn7). The transformed cells were cultured in Lutria Bertani (LB) medium (Sigma, St. Louis, Mo.) containing gentamicin 7.5 µg/mL, 47 µg/mL kanamycin, 10 µg/mL tetracycline, 100 µg/mL blue gal and IPTG 40 µg/mL, and the plates were incubated for 48 h at 37ºC.[24] The white colonies were isolated and subjected to PCR assay as follows: 3 min at 95ºC (35 cycles), 1min at 95ºC for denaturation, 1 min at 58ºC for primer annealing, 4 min at 68ºC for the extension, concluded with a final extension step at 68ºC for 10 min by using M13/pUC specific primers mentioned below:

M13/pUC Forward 5′-
CCCAGTCACGACGTTGTAAAACG-3′,
M13/pUC Reverse 5′-
AGCGGATAACAATTTCACACAGG-3′.

Insect Cells and Transfection

The insect cell *Sf9*, deriving from the *Spodoptera frugiperda* insect ovarian cell line, was utilized to produce recombinant protein by the *Baculovirus* expression system (National Center for Genetic and Biological Resources of Iran). The cell line was kept in Grace′s insect medium at 26ºC (Gibco, National Cell Bank Pasteur Institute of Iran). Insect cells were incubated as monolayers. After cell passages to obtain sufficient confluency (3×10^5 cells in each well of 6-well plate), the recombinant Bacmid were transfected into cells by electroporation method based on the optimized protocol (60 voltage, 600 µF capacitance). For confirmation of the transfection, the target gene (H1N1 HA) was amplified by the PCR technique and was sequenced using Sanger's DNA sequencing method.[26] The cells infected with recombinant Bacmid checked every day for cytopathic effects (CPE). After 96 h of incubation at 27°C, the flasks centrifuged at 3000 rpm for 5 min. Afterward, the supernatant containing rHA-expressing Baculoviruses collected and then transferred to 4°C refrigerator.

SDS-PAGE and Western Blotting Analyses

The Grace medium containing the infected monolayer cells were centrifuged at 3000 rpm for 5 min; the supernatant was collected and washed three times with cold PBS. Then the pellet was re-suspended in lysis buffer containing 300 mM NaCl, 1% Triton X-100, 20 mM Tris–HCl (pH 8), and 1X protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA) and incubated on an ice bath for 30 min. Protein was separated by 12% resolving gel at pH 8.8 and 4% stacking gel at pH 6.8 using the Mini-PROTEAN III system (Bio-Rad laboratories LtdAt). Firstly, the program was set to 100 mA for 30 min, and then it was run at 80 mA until the bromophenol blue reached the bottom of the gel (3 to 4 h). The concentration of rHA protein was measured by the Bradford method based on BSA standards. After detection, 15 µg of proteins were transferred to nitrocellulose membranes for western blotting assay.

Table 1. PCR primer used in this study

Primer	Sequence	Tm	CG%
HA Forward	GGTATTCACCATCCGAACGA	55°C	50%
HA Reverse	TTTCTTAACCAGCCAGATCAGGTTT	58°C	40%

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Western Blocking Solution was added to the western paper and placed it in a shaker for two hours. Then removed and rinsed with TBST 1X for two times. Afterward, the Anti His tag added to the papers and placed in darkness at 25°C for 1 hour on a slow shaker. The paper was rinsed two times with TBST 1X (0.05% Tween 20 in PBS) (firstly shacked for 15 min and then washed twice for 10 min on the shaker) followed by incubation with secondary antibody (HRP conjugated IgG (Sigma Aldrich). The membrane was removed and the TMB solution was added after washing 3 times with PBS/Tween20. The protein bands were visualized after 3 to 4 min by staining the membranes with diaminobenzidine.

Hemagglutination Assays of Recombinant Baculovirus

The HA test examines the ability of the influenza virus (serial dilution) to bind to molecules on the chicken's red blood cells (RBC). Briefly, the heparinized RBC suspension was washed with PBS via centrifugation at 1500 rpm for 7 min and 7°C three times. The RBC was prepared by PBS with 0.5% dilution. Two rows were selected in a macro-plate, and 50 µL of PBS was added into each well of 96 wells plate. 50 µL of the transfected cell was added to the first well and then serially diluted to the last well; and 50 µL from the last well was discarded at the end. Additionally, in the second row, the Influenza Inactivated antigen(as a control sample) was added and serially diluted in an equal volume of PBS. After finely mixing, the plates were incubated at room temperature for 30 min, and the titer of RBC agglutination was recorded. Moreover, the highest dilution well of the plate (first well) of HA was considered as a titer.

Purification and Formulation of HA Protein

The HA protein was expressed on the surface of the infected Sf9 insect cells and then purified by a Nickel-NTA gel column chromatography using immobilized metal affinity chromatography(IMAC) method.[27] Four milliliters of the lysate (containing 0.86 g NaH_2PO_4, 2.2 g NaCl, and 0.04 g Imidazole) was added to the resin and mixed gently by a shaker. The lysate/Ni-NTA mixture was placed on a shaker, at 4°C (25°C) for 2h and then packed into a column. The column was washed with 1.5 mL washing buffer (0.86 g NaH_2PO_4, 2.2 g NaCl, and 0.08 g Imidazole) and then eluted with the elution buffer containing 0.86 g NaH_2PO_4, 2.2 g NaCl, and 2.1 g Imidazole. All the above-mentioned steps were carried out on an ice bath. Finally, we verified and quantified the purified protein using SDS-PAGE and Bradford method, respectively. The target protein was formulated with the Mf59 adjuvant, to enhance immunogenicity. The recombinant protein and MF59 adjuvant were mixed at a ratio of 50/50; using a homogenizer.

Animals

In the current study, 45 healthy female BALB/c mice with 5 weeks of age were provided from the Razi Vaccine and Serum Institute of Iran (Karaj, Alborz, Iran). The mice were maintained in the Animal's Room (24ºC) of the Razi Vaccine and Serum Institute, and fed in 12-hour cycles (day and night). The animal experimental procedures were carried out following the ethics committee of Islamic Azad Tehran Medical Sciences (approval ID: IR.IAU.SHK.REC.1398.046).

Study Design, Experimental Groups, and Immunization

The 45 BALB/c mice were divided into three groups, each of which containing 15 individuals. The groups were as follows: i) vaccinated with recombinant protein plus MF59 adjuvant, ii) vaccinated with recombinant protein plus Alum adjuvant, iii) vaccinated with commercial H1N1 influenza vaccines; additionally, one group including 15 mice was considered as a negative control (non-vaccinated group). The mice were injected subcutaneously with an insulin syringe at 5 µg. The vaccination process was performed twice a day for 14 days. Two weeks after the second injection, 100 µL of the blood sample was collected by a pipette pasture from the dorsal lobe of the mice eye (cave retinal). The samples were then carefully transferred into a refrigerator at -6°C and maintained for 24 h.

Enzyme-linked Immunosorbent Assay (ELISA) for IgG

For this purpose, 275 µL of the purified protein was mixed with 725 µL coating buffer (0.0833 g Na_2CO_3, 0.144 g$NaHCO_3$, and 50 mL DDW), then every well was coated with 100 µL (5 µg/mL) of the mixture. Also, the protein-free buffer was added to some wells as the negative control. The plates were incubated overnight at 6°C. The plates were then blocked by adding a washing buffer containing BSA 1% for

60 min incubated at 37°C. Subsequently, the ELISA plates rinsed three times using a washing buffer. In the next step, 50 µL from each 1:10 and 1:100 of diluted serum concentration (final volume 100 µL) was added separately to each well of 96 wells plate and incubated at 25°C for 90 min. The plate was rinsed twice with the washing buffer and was mixed with 20 µL (1/100) of the antibody with 22 mL washing buffer. The mixture was added to the wells and incubated in a 37°C for 1 h. The washing process was performed twice again. A 100 µL of 3,3′,5,5′-Tetramethylbenzidine (TMB) was then added to each well and the plate was incubated for 15 min in darkness. After sufficient color development, 100 mL of stop solution (2.65 mL H_2SO_4, 47.35 mL DDW) added to all wells, and the absorbance immediately measured by an ELISA plate reader at 450 nm.

Hemagglutination Inhibition (HI) Assay

The nonspecific HA activity in hen sera was verified using 0.25% chicken RBCs. The HA elimination was performed with hem-adsorption against 4% chicken RBCs (final concentration) for 1 h at 4°C. The virus inoculum was back-titrated to verify the accuracy of the HA units before the HI test. Preliminary, 25 µL PBS buffer was loaded into each well, except the first one. Then, Receptor Destroying Enzyme (RDE)-treated serum was serially diluted 2-fold from 1:10 to 1:1280. Then, 25 µL from diluted serum was incubated with each well with 25 µL of test virus (4 HA unit) for 60 min at room temperature. After that, 50 µL of 0.5% chicken RBCs were added to the solution, and the reaction mixture was incubated at room temperature with a further 60 µL. Next, wells were investigated for inhibition of HA visually, in which well-defined RBC "buttons" or teardrop formation appeared upon the plate tilting as the index. HI titers were the reciprocal of the highest dilution of serum that completely prevented HA.

Influenza Virus Challenging

Two weeks after the last immunization, five female BALB/c mice from each group were infected intranasally with 5x104 PFU of Mouse adapted Influenza A/PR8/34(H1N1) stock, (prepared by Razi Vaccine & Serum Research Institute). Daily monitoring performed for disease symptoms. Two weeks later, the mortality rate of the animals was calculated. Any signs of the disease, body weight, and temperature also recorded. For determination of LD_{50}, 4 mice in 6 groups were infected intranasally by 50×10^{1}-50×10^{6} TCID viruses per 50 µL and after 2 weeks the final titer was determined as LD50, referring to the lethal dose for 50% of the population.

Statistical Analysis

After completing each experiment, the raw data recorded and the normality test evaluated by the Kolmogorov-Smirnoff test. The one-way analysis of variance (ANOVA) was used to analyze differences between the examined groups, and the level of statistical significance was considered at $p<0.05$. Post-hoc and Tukey tests using the Social Sciences software (SPSS, Inc., Chicago, IL, USA) version 20 were performed between groups. In addition, the mortality rate was analyzed by Kaplan-Meier log-rank test.

RESULTS

Cloning and Construction of Recombinant Bacmid

The considered band 654bp showing *HA* gene was detected on 1% gel electrophoresis (Figure 1). The gene of interest and pFastBac HT A vector were digested by two *XhoI* and *BamHI* enzymes (Figure 2A).

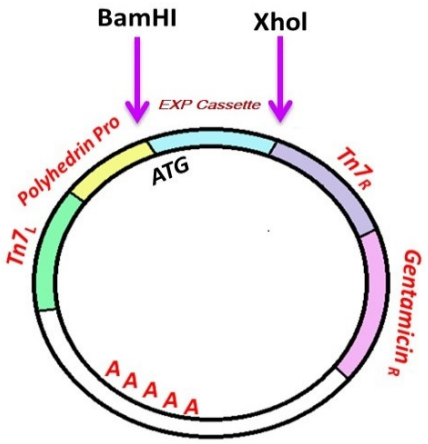

Figure 1. An illustration of the designed pFastBac HT A vector harboring the expression gene encoding H1N1 HA protein. The considered gene and vector were digested with two *XhoI* and *BamHI* restriction enzymes.

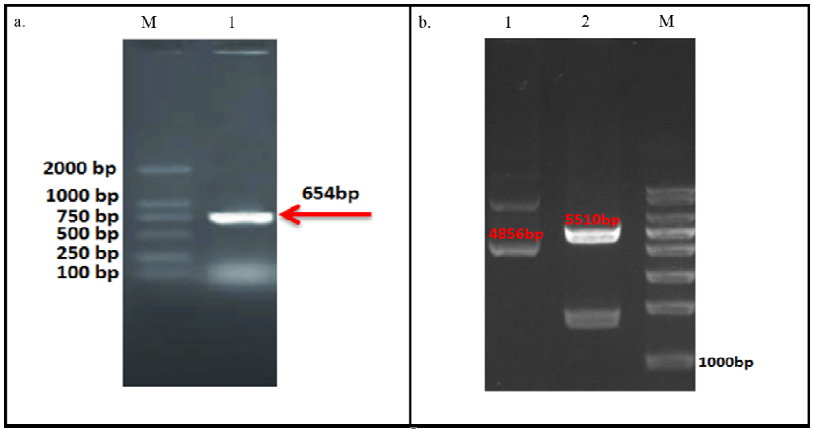

Figure 2. Cloning and construction of the hemagglutinin (HA) expression vector. a: 654bp length amplified by PCR from H1N1 cDNA (1). b: Restriction digestion of cloned vector. Lane 1: Digestion of pFast Bac HT A without the gene (4856 bp), Lane 2: Digestion of cloned vector by *XhoI* and *BamHI* (5510 bp). M: Ladder.

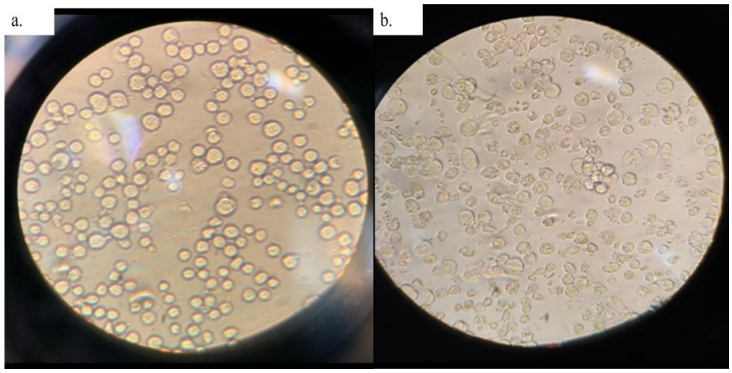

Figure 3. Morphological differentiation of insect cells after transfection. a: Sf9 cells before transfection, b: transfected Sf9 cells with recombinant bacmid after 96 h. Transfected cells are larger with impaired wall and have low density after 96 h.

After an overnight, the reaction was loaded on agarose gel 1% and confirmed the exact 4865bp band. The gel recovery was carried out and the concentration of the vector and HA gene was 50 and 34.7 ng/µL, respectively. The ligation was performed according to the protocol, then cloned into *E.coli DH5α*. Final validation was performed by double digestion with two *Xho*I and *BamH*I restriction enzymes and then visualized by gel electrophoresis (Figure 2B). Afterward, Sf9 cell transfected with recombinant bacmid and the transfection monitored by microscope for 96 h. The big shape, low density, wall disruption, and suspension of cells demonstrated the transfection (Figure 3).

SDS-PAGE Analysis and Western Blotting

In the SDS-PAGE gel, different samples including *Baculoviruses* infected cells, supernatants, uninfected cells and H1N1 influenza virus were gel stained (Figure 4A). Accordingly, the estimated molecular weight of the recombinant protein (˷60 kDa) was observed. Moreover, the separated proteins on SDS-PAGE were transferred to paper and the specificity of the rHA was assessed by western blot assay. Based on figure 4B, the rHA protein probed with anti-His tag and monoclonal antibodies HRP conjugate (IgG) was detected, which further confirms the validation of rHA expression.

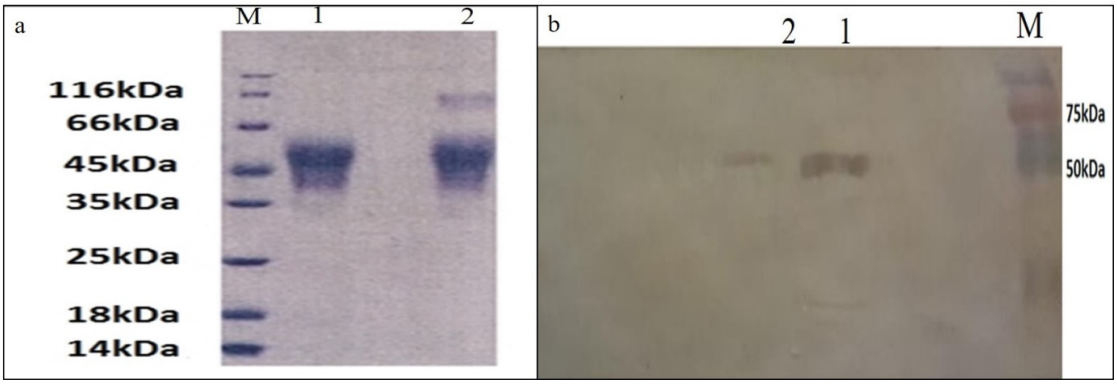

Figure 4. SDS-PAGE and Western blot results for proteins produced in Sf9 cells. a: M: ladder, 1: positive control, 2: sample hemagglutinin (HA) protein. b: M: ladder, 1: positive control, 2: HA protein

The protein band is observed between the protein ladder bonds of 66-45 kDa equal to 60 kDa, indicating the exact size of the recombinant protein (Figure 4B).

Hemagglutination Test Result

The HA test was carried out in 25 µL from two-fold serially diluted virus-containing Sf9 cells and determined based on the detection of agglutination in 1:256 dilution (Figure 5). The results were indicated the proper attachment of the HA ligand on the surface of the cells and confirmed the bioactivity of the HA. It should also be noticed that the neutralization activity of the specific antibodies raising after immunization.

Evaluation of Immune Responses

HI and ELISA-induced humoral immunity were evaluated for antibody responses 2 weeks after the last vaccine injection. Serum HI antibody titers against three groups of vaccines were determined from serum samples collected after 2 weeks of immunizations and the mean titers obtained by this assay are shown in Figure 4A. Immunization of mice with MF59+rHA induced the high HI antibody titers (512) against A/PR8/34/H1N1 strain. The HI antibody titer against MF59+rHA was significantly greater than the Alum+rHA and the vaccine, respectively (Figure 6A). Additionally, the antibody reactivity to influenza virus strains was measured through the strain-specific serum antibody titers of sera used for HI assays. The results showed that all groups immunized by recombinant protein (rHA + alum; rHA + MF59), as well as commercial TIV, boosted the antibody production against the virus as compared to the negative control (Figure 6B). Notably, the Mf59 significantly enhanced the humoral response to 1/10 titer rHA antigen with adjuvant activity comparable to those of Alum and TIV vaccine. Simultaneously, the neutralizing antibody decreased against a HA antigen by dilution titer to 1/100 in all groups (Figure 5B). It is also worthwhile to note thatMF59 induced a more neutralizing antibody as compared to Alum and TIV.

Influenza Virus Challenging

Upon challenge with the H1N1 virus, the vaccinated groups with MF59 formulated with rHA showed a slight weight loss and mortality rate (92%); however, the Alum and TIV vaccinated mice lost more than 10% of their initial body weight and the mortalityincreasedby8 days post-infection (Figure 7A and B). Furthermore, body temperature increased severely in mice vaccinated by MF59 (39.2°C) by 6 days post-infection compared with Alum (38.4°C) and TIV (38.8°C) groups (Figure 7C).

Therefore, it is clear that target delivery of MF59 is more than Alum and TIV and this may be due to the high stability and strong binding with HA antigen. Also, recombinant H1N1 HA with MF59 adjuvant caused 92% immunity within 10 days in infused mice against infection. The animals of control groups were died 6 days after the first infection.

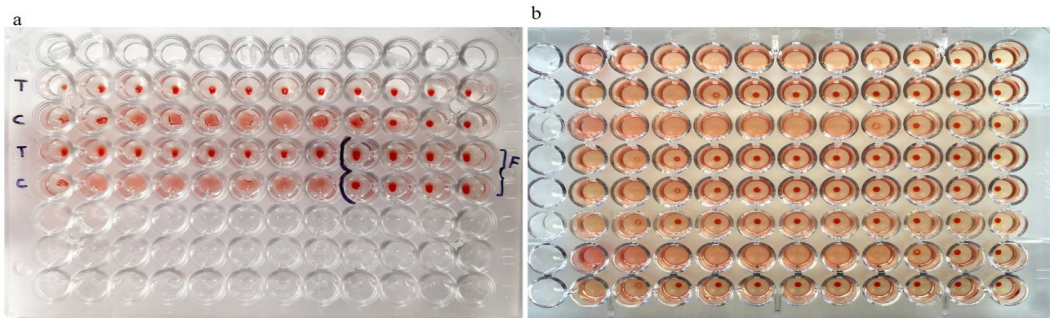

Figure 5. A: Hemagglutination and B: hemagglutination inhibition (HI) assays of H1N1 influenza viruses using chicken RBCs. In the hemagglutination assay, the treatment and control samples were indicated with "T" and "C" letters.

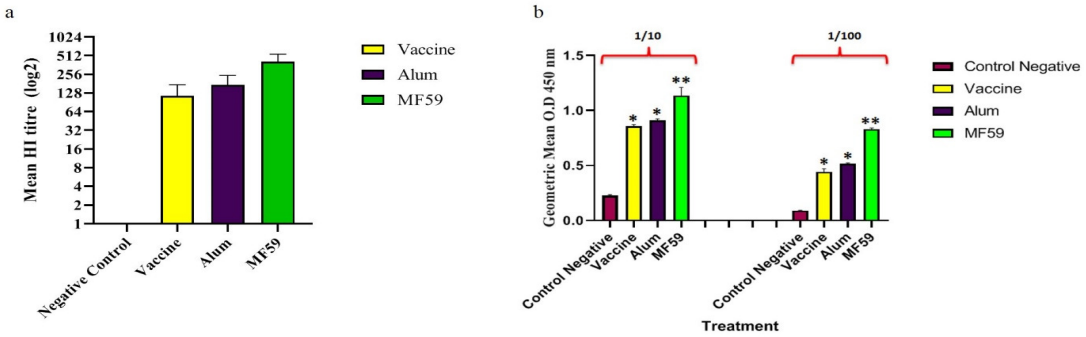

Figure 6. Results of ELISA and hemagglutination inhibition (HI) test for influenza viruses A/PR8/34(H1N1). At two weeks after the last immunization, the HI antibody titer (total IgG) boosted against 25 µL MF59+rHA significantly (A), also the geometric mean determined by ELISA was significantly different between three vaccine groups in both dilution (1:10 and 1:100). The MF59+rHA induced immune response compare to Alum and commercial vaccine with a significant relationship (B).

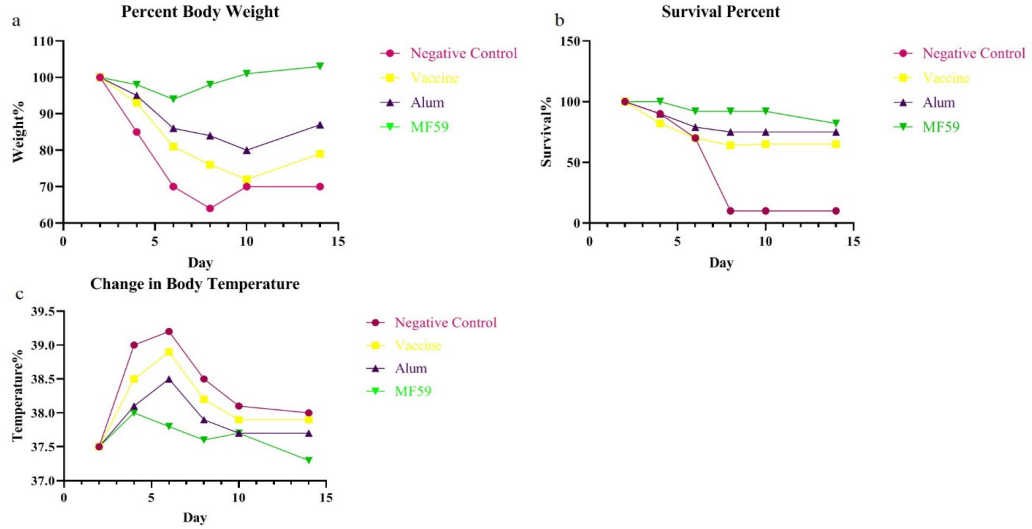

Figure 7. Challenges in the protection of BALB/c mice against influenza virus. Mice were infected intranasally with a lethal dose (5x10^4 PFU) of influenza virus A/PR8/34(H1N1), and monitored daily for 2 weeks. A: Body weight change, B: survival percent and C: body temperature was evaluated after challenging

DISCUSSION

Production of recombinant antigens, like the influenza vaccine, allows a more timely response to a potential influenza pandemic. In fact, due to the rapid changing of the virus genome, the previous vaccines can not control the pandemic.[23] These vaccines also cause a poor immune response, expensive, and need to reconstitute every year. Therefore, making a safe, effective, and affordable vaccine that considerably boosts the humoral and cellular immune responses is essential. HA is the most important surface protein of the influenza virus, which can stimulate a wide range of immune responses against the influenza virus and producing high titers of antibodies.[28] HA is the main antigen of this virus against neutralizing antibodies, and influenza virus epidemics are based on changes in its antigenic structure.[28,29] Unlike the upper HA region; the stem of the molecule is antigenically stable and less mutated.[29,27] Nowadays, genome sequencing and recombinant DNA technology have provided scientists the opportunity to replace new antigens with previous ones, thereby accelerating the production of new vaccines.[29] Altogether, taking advantage of the recent technology, as well as advanced molecular methods, can have our ways toward developing an efficient vaccine against influenza virus. Therefore, herein we developed a novel vaccine against the H1N1 virus with low side-effects and significant antibody production.

Currently, the Baculovirus-insect cell expression system has been extensively utilized for the production of complicated proteins, particularly for viral and parasitic antigens as commercial manufacturing technology. In this study, the eukaryotic expression vector was used to produce the rHA protein. The main advantage of the eukaryotic expression systems compared to the prokaryotic systems is post-translational modifications (PTMs) such as disulfide bonds formation, glycosylation, phosphorylation, and oligomerization. So, it can be assured that the properties of the produced protein are especially similar to the protein produced by the virus.[30] The vector used in this study has a strong PUC replication origin and is capable of replicating in DH10. Also, it can replicate in eukaryotic cells because of its SV40 replication origin. Gene expression also occurs under a strong polyhedrin promoter. It is important to highlight the fact that the protein expressed by this vector is secreted by the cells due to the mouse kappa immunoglobulin chain sequence.

The results of this study indicate that the synthesized recombinant plasmid sufficiently produces and secretes this gene product under *in vitro* conditions. The identification of this protein by the H1N1 monoclonal antibody in the HI test (Figure 5) indicates that the rHA can stimulate the immune system to produce neutralizing antibodies. One of the main obstacles in producing human vaccines is the remarkable demands for the vaccine and its components, as the requests for such vaccines increasingly grow. One approach to solve this problem involves the use of different adjuvants or cytokines. In this study, the Bac-to-Bac system was applied to produce HA antigen in insect cells. In 2006, Wang and colleagues also examined the expression and purification of HA protein in an insect cell,[31] but we used the Iranian strain of the influenza virus in this study, and the expression system in this study was Bac-to-Bac, which ultimately increased purity and stability of the recombinant protein. These results of the present work and future studies could eventually be a good alternative to the influenza vaccine in Iran. The Baculovirus expression system was successfully used to synthesize multiple virus capsids, including HCV-like particles (VLPs) in the insect cell by Baumart et al. in 1998. In 2006, by using this system, Hu YC and co-workers expressed two HA genes of the H5N2 strain in an insect cell.[32] Herein, the rHA proteins used as alternative antigen standards for pandemic influenza vaccines.[33] Also, Huang et al (2017) evaluated the antibody responses to trivalent inactivated influenza vaccine in health care personnel previously vaccinated and with getting vaccinated for the first time.[34] The SDS-PAGE results showed that inoculation of insect cells with recombinant Baculovirus with MOI equivalent to 10 and harvesting of cells 72 hours after an infection led to the production of recombinant protein.

Evaluation of antibody responses showed that the recombinant vaccine and rHA injection were successful in inducing humoral responses. The results showed that all immunized groups with recombinant vaccine and HA or inactivated influenza virus significantly induced antibodies against the H1N1 strain compared to the negative control group. Nonetheless, the formulation of Mf59 and rHA significantly boosted the antibody titers as compared to Alum and TIV vaccine. Furthermore, immunogenicity and efficacy evaluations demonstrated

that the developed oil-based vaccine candidate (Mf59+rHA) was more potent than a commercial vaccine. Previously, the potent of the Alum adjuvant against the influenza virus showed that this candidate can slightly enhance immunogenicity;[35] however, the application of the MF59 adjuvant enhances the humoral and cellular responses, which indicates to the importance of the adjuvant in future high-efficiency influenza vaccine production. As previously demonstrated, utilizing MF59 enhances the functional antibody responses to HA-based vaccines quantitatively and qualitatively by promoting both epitope breadth and binding affinity.[36] Assessment through viral challenge further confirmed the immunogenicity of the candidate vaccine. The results showed that likewise commercial vaccine, the Mf59 + rHA formulated could effectively protect the animals against the H1N1 virus (with a 92% survival rate). However, the mortality rate in the control group of mice was significantly increased and they lost their body weight after six to eight days post-infection. Collectively, our results clearly illustrate the safety and efficiency of a novel vaccine candidate against the H1N1 virus in comparison with the commercial vaccine. Additionally, the production of rHA protein using *sf9* insect cells and Bac to Bac expression system can considerably facilitate scaling-up the required protein.

In conclusion, previous researches focused on the initiation of a seasonal vaccine against influenza viruses, and in this research, an HA-protected protein vaccine in the form of a monocistronic vector was proposed as an effective strategy to create immune responses against influenza virus. For expression in a eukaryotic secretory system, pFastBac HT A vector was used and the results were confirmed by HA and western blot analysis. Subsequently, mice were injected and compared with the commercial vaccine, split virus vaccine, and also with the control group produced a favorable level of antibody. The antiserum from animals evaluated by HI and ELISA tests, not only made it possible to neutralize the H1N1 strains but also led to the survival of infected mice with an acute strain of H1N1 and the results were comparable to the commercial vaccine recipient group. The results also showed the superiority of the vaccine produced in the Sf9 insect cell to the vaccine produced in the egg. Also, the recombinant protein combined with the MF59 adjuvant is safer than the Alum adjuvant and in future studies, it can be a reliable vaccine against the H1N1 virus.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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REFERENCES

1. Noda T, Kawaoka Y. Classification and virion structure of influenza virus. Jpn Clin Med. 2006;64(10):1766-9.
2. Wright P, Neumann G, Kawaoka Y. Chapter 48 Orthomyxoviruses. Fields Virology Fifth Edition Philadelphia, PA, USA: Lippincott Williams & Wilkins, 2007:1691-740.
3. Cox NJ, Tamblyn SE, Tam T. Influenza pandemic planning. Vacc. 2003;21(16):1801-3.
4. Kilbourne ED. Influenza pandemics of the 20th century. Emerg Infect Dis. 2006;12(1):9.
5. Ford SM, Grabenstein JD. Pandemics, avian influenza A (H5N1), and a strategy for pharmacists. Pharmacother. 2006;26(3):312-22.
6. Gendon I. Influenza pandemic: Hypotheses and facts. Zh. Mikrobiol Epidemiol Immunobiol. 2008;5:109-18.
7. Kitikoon P, Vincent AL, Janke BH, Erickson B, Strait EL, Yu S, et al. Swine influenza matrix 2 (M2) protein contributes to protection against infection with different H1 swine influenza virus (SIV) isolates. Vaccine. 2009;28(2):523-31.
8. Zangwill KM, Belshe RB. Safety and efficacy of trivalent inactivated influenza vaccine in young children: a summary for the new era of routine vaccination. Pediatr Infect Dis J. 2004;23(3):189-97.
9. Frolov VG, Seid Jr RC, Odutayo O, Al Khalili M, Yu J, Frolova OY, et al. Transcutaneous delivery and thermostability of a dry trivalent inactivated influenza vaccine patch. Influenza Other Resp. 2008;2(2):53-60.

10. Watanabe T, Watanabe S, Kim JH, Hatta M, Kawaoka Y. Novel approach to the development of effective H5N1 influenza A virus vaccines: use of M2 cytoplasmic tail mutants. J Virol. 2008;82(5):2486-92.
11. Ebrahimi SM, Tebianian M, Toghyani H, Memarnejadian A, Attaran HR. Cloning, expression and purification of the influenza A (H9N2) virus M2e antigen and truncated Mycobacterium tuberculosis HSP70 as a fusion protein in Pichia pastoris. Protein Expr Purif. 2010;70(1):7-12.
12. Kawaoka Y. Influenza virology: current topics. Caister Academic Press; 2006.
13. Maeda Y, Horimoto T, Kawaoka Y. Classification and genome structure of influenza virus. Jpn Clin Med. 2003;61(11):1886-91.
14. Mair CM, Ludwig K, Herrmann A, Sieben C. Receptor binding and pH stability — How influenza A virus hemagglutinin affects host-specific virus infection. Biochim Biophys Acta Biomembr. 2014;1838(4):1153-68.
15. Bobone S, Hilsch M, Storm J, Dunsing V, Herrmann A, Chiantia S. Phosphatidylserine lateral organization influences the interaction of Influenza virus Matrix Protein 1 with lipid membranes. J Virol. 2017;91(12):e00267-17.
16. Sedova E, Shcherbinin D, Migunov A, Iu LD, Shmarov M, Tsybalova L, et al. Recombinant influenza vaccines. Acta Nat. 2012;4(15):17-27.
17. Smits A, Hak E, Stalman W, Van Essen G, Hoes A, Verheij TJ. Clinical effectiveness of conventional influenza vaccination in asthmatic children. Epidemiol Infect. 2002;128(2):205-11.
18. Demoly P, Dhivert-Donnadieu H, Bousquet J. Vaccination with allergens in children. Allergy Immunol. 2000;32(10):397-401.
19. Perrie Y, Mohammed AR, Kirby DJ, McNeil SE, Bramwell VW. Vaccine adjuvant systems: enhancing the efficacy of sub-unit protein antigens. Int J Pharm Pharmacol. 2008;364(2):272-80.
20. Girard MP, Katz JM, Pervikov Y, Hombach J, Tam JS. Report of the 7th meeting on evaluation of pandemic influenza vaccines in clinical trials, World Health Organization, Geneva, 17–18 February 2011. Vaccine. 2011;29(44):7579-86.
21. Soleimanjahi H, Fotouhi F. Baculoviruses and insect cells as powerful tools for gene expression. 2009: Tehran: Jahad Daneshgahi Publication. Tehran, Iran. 5-45.
22. Hwang JH, Lee SW, Rha SJ. Dietary green tea extract improves growth performance, body composition, and stress recovery in the juvenile black rockfish, Sebastes schlegeli. Aquac Int. 2013;21(3):525-38.
23. Dormitzer PR, Suphaphiphat P, Gibson DG, Wentworth DE, Stockwell TB, Algire MA, et al. Synthetic generation of influenza vaccine viruses for rapid response to pandemics. Sci Transl Med. 2013;5(185):168-85.
24. Sambrook J, Russell DW. Molecular Cloning: A Laboratory Manual, 3rd Edition (2001), Cold Spring Harbor Laboratory Press.
25. Zadeh SH, Fotouhi F, Kheiri MT, Razavi MR, Heydarchi B, Farahmand B, et al. Isolation and cloning of large subunit of Influenza virus A (H1N1) hemagglutinin gene into Bacmid vector to construct recombinant Baculovirus. In BMC Proc. 2011;5(1):1-1.
26. Smith LM, Hood LE, Hunkapiller MW, Hunkapiller TJ, inventors; California Institute of Technology, assignee. Automated DNA sequencing technique. United States patent US 5,171,534. 1992.
27. Abe R, Kudou M, Tanaka Y, Arakawa T, Tsumoto K. Immobilized metal affinity chromatography in the presence of arginine. Biochem Biophys Res Commun. 2009;381(3):306-10.
28. Kreijtz J, Fouchier R, Rimmelzwaan G. Immune responses to influenza virus infection. Virus Res. 2011;162(1-2):19-30.
29. Yang S, Lee JY, Lee JS, Mitchell WP, Oh HB, Kang C, et al. Influenza sequence and epitope database. Nucleic Acids Res. 2009;37(suppl_1):423-30.
30. Thakur R, Shankar J. Strategies for Gene Expression in Prokaryotic and Eukaryotic System. InMetabolic Engineering for Bioactive Compounds Springer, Singapore. 2017:223-47.
31. Wang K, Holtz KM, Anderson K, Chubet R, Mahmoud W, Cox MM. Expression and purification of an influenza hemagglutinin--one step closer to a recombinant protein-based influenza vaccine. Vaccine. 2006;24(12):2176-85.
32. Hu YC, Luo YL, Ji WT, Chulu JL, Chang PC, Shieh H, et al. Dual expression of the HA protein of H5N2 avian influenza virus in a Baculovirus system. J Virol Methods. 2006;135(1):43-8.
33. Choi Y, Kwon SY, Oh HJ, Shim S, Chang S,

Chung HJ, et al. Application of recombinant hemagglutinin proteins as alternative antigen standards for pandemic influenza vaccines. Biotechnol Lett. 2017;39(9):1375-80.

34. Huang KYA, Chang SC, Huang YC, Chiu CH, Lin TY. Antibody responses to trivalent inactivated influenza vaccine in health care personnel previously vaccinated and vaccinated for the first time. Sci Rep. 2017;7(1):1-10.
35. Jafari M, Moghaddam Pour M, Taghizadeh M, Masoudi S, Bayat Z. Comparative assessment of humoral immune responses of aluminum hydroxide and oil-emulsion adjuvants in Influenza (H9N2) and Newcastle inactive vaccines to chickens. Artif Cells Nanomed Biotechnol. 2017;45(1):84-9.
36. Khurana S, Verma N, Yewdell JW, Hilbert AK, Castellino F, Lattanzi M, et al. MF59 adjuvant enhances diversity and affinity of antibody-mediated immune response to pandemic influenza vaccines. Sci Transl Med. 2011;3(85):48-85.