Iranian Journal of Virology 2017;11(4): 31-38

Original Article

Production and Evaluation of Polyclonal Antibody Against Influenza A Virus Matrix 2 Conserved Protein for Research and Diagnosis Purposes

Zamani S[1,2], Fotouhi Chahouki F[1], Mehrbod P[1], Sadeghi Neshat S[1], Farahmand B[1*]

1. Department of Influenza and other Respiratory Viruses, Pasteur Institute of Iran, Tehran, Iran.
2. Department of Biology, Science and Research Branch, Islamic Azad University, Tehran, Iran.

Abstract

Background and Aims: The aim of this study was to produce and purify the Polyclonal antibody(pAb) against Matrix protein 2 (M2) with reasonable efficiency. Matrix protein 2 is one of the most conserved proteins of the influenza A virus which acts as ion channel. Polyclonal antibodyproduced against Matrix protein 2 is used in vaccine research, passive immunization and qualitative/quantitative analysis methods.

Materials and Methods: Recombinant M2 protein was produced in E.coli. Purified protein with Freund's adjuvants (Complete and Incomplete) was injected into two New-Zealand white male rabbits. The polyclonal antisera of rabbits were evaluated by RID, immuno-blotting and ELISA. The IgG was purified using DAEA-cellulose column chromatography. Finally, the quality and properties of purified IgG were evaluated using SDS-PAGE and ELISA .

Results: The RID and immunoblotting results showed that the produced anti-M2 antibody was able to recognize M2 recombinant protein epitopes. The ELISA results confirmed anti-M2 pAb reached reasonable titers after three injections. IgG against M2 was purified with suitable concentration. The Purified polyclonal IgG-M2 was evaluated using ELISA and the results showed IgG-M2 reacted with the antigen up to 1:32000.

Conclusions: The data showed that recombinant M2 protein was able to stimulate immune response to produce antibody at satisfactory level.

Keywords: ELISA, Influenza Virus, M2 protein, polyclonal antibody, RID.

Introduction

Influenza virus is one of the most significant agent constitutive for public health concerns during seasonal epidemics and usually children, elderly and people with chronic medical conditions are at peril (1).

Influenza virus belongs to the Orthomyxo-viridae family. Its genome consists of eight fragments of negative sense single strand RNAs. This virus is covered by lipid envelope and different proteins are spiked onto its surface. Influenza virus is classified into three subtypes A, B, and C. The subtype A infects birds, humans and other mammalians such as swine and horse. Influenza A virus causes respiratory acute disease in human and involves upper respiratory system. The most important proteins of this virus include neura-minidase (NA), hemagglutinin (HA), and matrix proteins (1 and 2) (2). Among them HA and NA endure the most changes through antigenic drifts and shifts. According to these changes, influenza A viruses are classified based on 16 HA (H1-H16) and 9 NA (N1-N9) glycoproteins (3). H1N1, H2N2, and H3N2 subtypes usually infect humans and lead to

[*] **Corresponding author:** *Behrokh Farahmand
Department of Influenza and other Respiratory Viruses, Pasteur Institute of Iran.
Tel: +98 21 64112130, Fax: +98 21 66496517
E-mail: b_farahmand@pasteur.ac.ir

seasonal epidemics every year. Sometimes, some strains such as H1N1 subtype could cause global pandemics and extensive morality worldwide (4).

In recent years, researchers mostly focus on conserved antigenic proteins such as matrix protein 2 (M2) for vaccine production, serological studies and polyclonal and monoclonal antibodies production (5, 6). M2 which was discovered by Robert Lambin in 1980is a homotetramer single pass membrane protein, this channel plays important role in releasing the virus genome by passing the proton through (7). M2 protein has 97 amino acids which consists of three domains, an extracellular N-terminal domain (23 a.a), a transmembrane domain (23 a.a) and an intracellular C-terminal domain (51 a.a) (2).

The most important M2 protein region is the extracellular N-terminal domain (M2e) which is highly conserved in a broad range of influenza A virus subtypes(7, 8). According to different researches, the M2 polyclonal antibodies can be used for influenza virus research in the field of vaccine production, development of diagnostic systems and serological studies (6, 9). The aim of this study was to produce anti-M2 polyclonal antibody with suitable quality for research and diagnostic purposes.

Methods

Physicochemical properties prediction. Recombinant M2 protein was produced as previously described using pET vector in prokaryotic expression system in our lab (5). The physico-chemical properties of recombinant M2 protein (such as aliphatic index, grand average of hydropathicity (GRAVY), instability index, estimated half-life and pI) were predicted using ExPASY-ProtParam tool. These properties are important for protein purification and adjuvant selection for immunization (10).

M2 protein immunogenicity prediction.

There are many tools for immunogenicity prediction such as Ellipro, DiscoTope and etc, ElliPro is a web-tool which perform using the MODELLER program and the JSmol viewer, it predicts and visualizes antibody epitopes in a submitted protein sequence or structure (11).

Animals' immunization. For polyclonal antibody production, two New-Zeland white male rabbits were immunized using mixture of purified recombinant M2 protein (300µg/ml) and equal volume of Complete Freund's adjuvant (Sigma Company) (CFA). Each injection was performed subcutaneously. Four weeks later, rabbits were boosted with a mixture of purified recombinant M2 protein (150µg/ml) and equal volume of Incomplete Freund's adjuvant (Sigma Company) (ICFA). Marginal ear vein blood was collected periodically every week before each booster injection and sera were generated following defibrillation. Rabbits were maintained for 6 months of time and were periodically boosted with M2 protein to maintain the anti-M2 titer.

Immunoassays for M2 antiserum evaluation. M2 antisera were evaluated by immunoassay techniques such as: double and single radial immunodiffusion (RID), immune blotting analysis (Western blot and dot blot) and ELISA.

Radial immunodiffusion(RID) assays. For this purpose, 2% agarose gel was prepared in PBS (8 g NaCl, 0.2 g KCl, 1.14 g NaH2PO4, 0.2 g KH2PO4, water) and solidified gel was punched to create wells on the gel. Central well was filled with optimal dilution of antigen and around wells were filled using serial dilutions of antiserum. The gel was incubated at 25°C in a humid chamber for 48 hours to allow diffusion of the antigens and antibodies.

Following incubation, gels were washed sequentially with a saline solution (0.9% NaCl) and distilled water, then dried and stained with Coomassie Blue (0.5% w/v).

For single radial immune diffusion (SRID) assay, to prepare 2% agarose gel in PBS, the optimal amount of anti-M2 antiserum was added before gel solidification. In order to make four millimeter wells, solidified gels were punched. Antigen was serially diluted in PBS. Dilutions of antigen were loaded sequentially onto the agarose gel wells and gels were incubated in a humidified chamber and washed and stained as double radial immune diffusion (DRID). All dried gels were scanned for image

evaluation. Standard agarose gels were 3 mm thick; however, in some experiments, thin gels (i.e., 2.2 mm) were prepared to facilitate better visualization of the precipitin rings (12).

SDS-PAGE and protein blotting assays. For Western blot analysis of anti-M2 antiserum, recombinant M2 protein was electrophoresed on 15% sodium dodecyl sulfate poly acrylamid gel in denatured condition, then protein bands were transferred to nitrocellulose membrane (by APPLEX semi-dry,10mA). After blocking the membrane with 1.5% BSA (1.5 g BSA, 100ml PBS), suitable dilution (1:100) of rabbit antiserum was added, then appropriate dilution of Goat Anti-Rabbit HRP-Conjugated (1: 50000) (Sigma-Aldrich, Taufkirchen, Germany) was added as secondary antibody. Finally, M2 band was visualized using Diamino benzoic acid (DAB) substrate.

For dot blotting procedure, recombinant M2 protein and 3M2e-HA2 chimeric protein (which contains critical M2 antigenic epitope) were directly placed on nitrocellulose membrane and other steps were conducted similar to Western Blot assay.

Enzyme Linked Immunosorbent Assay (ELISA).

The indirect ELISA was performed for comparison of rabbit antiserums titers. In brief, 96-well microtiter plates were coated with M2e synthetic peptide (conserved antigenic site of M2 protein) working solution (0.01µg/100µl). The plates were covered and incubated overnight at 4. After removing the extra coating solution, plates were washed with PBS Tween-20(0.05%) and blocked with 3% BSA (3g BSA, 100ml PBS). Then serial dilutions of rabbit antiserum solutions were added to each well and incubated for 2 hours at room temperature. Washing steps were repeated. In the next step, washed plates were treated using anti-rabbit HRP-conjugated secondary antibody. Finally, the enzymatic reaction was developed with 3, 3', 5, 5' tetramethylbenzidine (TMB) substrate at 450 nm wavelength.

Table 1. physicochemical properties of recombinant M2 protein determined by ProtParam

M2 recombination acid amino Sequence	MGSSHHHHHHSSGLVPRGSHMASMTGGQQMGRGSMSLLTEVETPIR NEWGCRCNDSSDPLVVAASIIGIVHLILWIIDRLFSKSIYRIFKHGLKRGP STEGVPESMREEYREEQQNAVDA DDGHFVSIELKKLAAALEHH HHHH
Number of amino acids	144
Molecular weight	16121.1
Theoretical pI	6.58
Total number of negatively charged residues (Asp + Glu)	17
Total number of positively charged residues (Arg + Lys)	14
Formula	C699H1093N217O208S8
Estimated half-life	30 hours (mammalian reticulocytes, in vitro)
	>20 hours (yeast, in vivo)
	>10 hours (Escherichia coli, in vivo)
Instability index	The instability index (II) is computed to be 31.14
	This classifies the protein as stable
Aliphatic index	78.54
Grand average of hydropathicity (GRAVY)	-0.478

Table 2. M2 predicted epitopes (A) Linear (B) Discontinuous

(A)Predicted linear epitopes

No	Start	End	Peptide	Number of residue	Score
1	133	144	LAAALEHHHHHH	12	0.922
2	114	127	QQNAVDADDGHFVS	14	0.652
3	1	19	MGSSHHHHHHSSGLVPRGS	19	0.646
4	41	49	VETPIRNEW	9	0.641
5	89	101	FKHGLKRGPSTEG	13	0.619
6	25	33	TGGQQMGRG	9	0.508

(B) predicted discontinuous epitopes

No	Residue	Number of residue	Score
1	_:Q114, _:Q115, _:N116, _:A117, _:V118, _:D119, _:A120, _:D121, _:D122, _:G123, _:H124, _:F125, _:V126, _:S127, _:E129, _:L130, _:L133, _:A134, _:A135, _:A136, _:L137, _:E138, _:H139, _:H140, _:H141, _:H142, _:H143, _:H144	28	0.743
2	:E40, :V41, _:E42, _:T43, _:P44, _:I45, _:R46, _:N47, _:E48	9	0.66
3	_::F89, _:K90, _:H91, _:G92, _:L93, _:K94, _:R95, _:G96, _:P97, _:S98, _:T99, _:E100, _:G101, _:E108, _:E109, _:R111, _:E112	17	0.623
4	:M1, _:G2, _:S3, _:S4, _:H5, _:H6, _:H7, _:H8, _:H9, _:H10, _:S11, _:S12, _:G13, _:L14, _:V15, _:P16, _:G18, _:S19, _:M24, _:T25, _:G26, _:G27, _:Q28, _:M30, _:G31, _:R32, _:G33, _:M35	28	0.599
5	_:C53, _:N54, _:D55	3	0.563

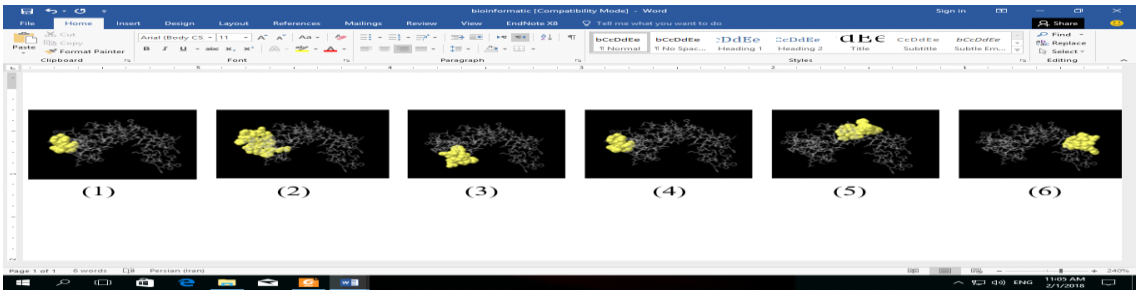
Antibody purification. For purification of IgG from confirmed antiserum, the proteins in antisera were precipitated using (36%) ammonium sulfate. The pellet was dissolved in phosphate buffer (1.6 g NaH2PO4, 0.516 g Na2HPO4, 17mM, pH6.3) and dialyzed against phosphate buffer overnight at 4°C.
Finally, the ɣ globulin containing antibody was purified using DAEA-cellulose column chromatography and its concentration was measured by Bradford assay. The purity of polyclonal antibody was analyzed using SDS-PAGE (12% gel) and efficiency of anti-M2 IgG was determined by ELISA.

Results

Prediction of physico-chemical properties. The result of physicochemical properties of M2 recombinant protein is shown in Table 1. The estimated parameters were useful for protein handling and application. The instability index was used to determine whether it will be stable in a test tube. The computed instability index for recombinant M2 protein is 31.14 which is less than 40, thus it is probably stable in the test tube. The aliphatic index of a protein is defined as the relative volume occupied by aliphatic side chains (alanine, valine, isoleucine, and leucine). It may be regarded as a positive factor for the increase of thermostability of globular proteins. The aliphatic index of this protein showed that it is a thermostable protein (13).
The negative GRAVY score meant that the protein is nonpolar and it is mixed to nonpolar phase of oil in water such as Freud's adjuvant. The predicted molecular weight and pI are

important for protein detection and purification steps (14).

A



B

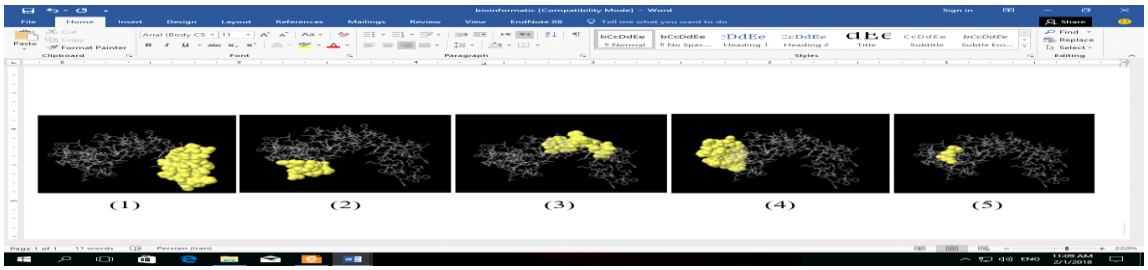


Fig. 1. JSmol 3D structure view rendered of recombinant M2 and antigenic epitopes. (A) linear epitops, (B) discontinuous epitops (In the ball-and-stick model, yellow balls are the residues of predicted epitopes and white sticks are the structures for non-epitope and core residues).

M2 protein immunogenicity prediction. In ellipro Web Tool, the molecular viewer JSmol was used to visualize linear and discontinuous epitopes on the M2 protein 3D structure. The results of linear and discontinuous epitopes visualization are shown in Table 2 and Fig 1.
As seen in Figure 1 (A&B) and Table 2 (A&B) most parts of M2 epitopes considered as linear epitopes for B-cells and predictions show that most of these epitopes are accessible in the surface of M2 proteins.

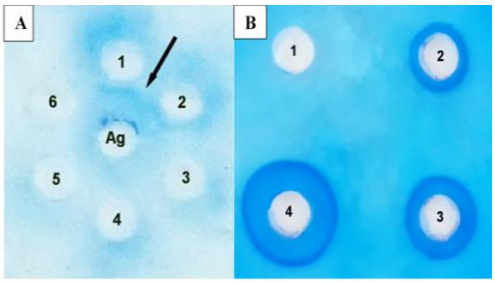


Fig. 2. Radial ImmunoDiffussion evaluation results (A) DRID.2.3% gel, Ag: M2 Protein, Antiserum Dilutions (wells 1&2): 1/8; (well 3): 1/16;(wells 4 & 5): 1/32; (well 6): Negative control, (B) SRID. 2% gel, Antiserum/gel (30 µl /ml); (well 1): Negative control, (well 2): M2 Protein (40 µl), (well 3); M2 Protein (80 µl) ;(well 4): M2 Protein (160µl).

Radial Immunodiffusion (RID) assay. In Double RID, interaction between antiserum and the antigen leads to observation of the sedimentary line up to 1.8 dilution of antisera (Fig. 2A). Also, sedimentary zones with high density were observed in SRID (Fig. 2B).
Immune blotting. To evaluate of M2 polyclonal antibody was performed immune blotting analysis that its results were positive for M2 and 3M2e-HA2 (Fig. 3 & 4).

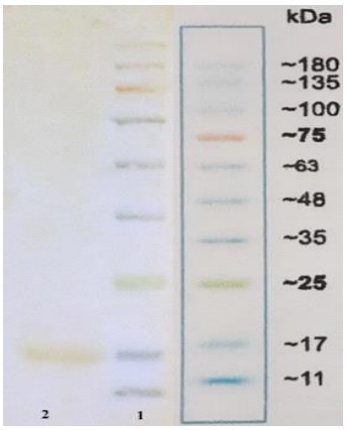


Fig. 3. Western blotting result for anti-M2 antiserum evaluation: lane 1: Ladder; lane 2: recombination M2 protein.

Fig. 4. Dot blotting result. dot 1: M2 recombination protein; dot 2: 3M2e-HA2 Chimer protein.

ELISA and evaluation of purification. The comparison of rabbits' antisera ELISA results showed that the amounts of antibodies against recombinant M2 protein were raised during 4 - 7 weeks (Fig. 5).

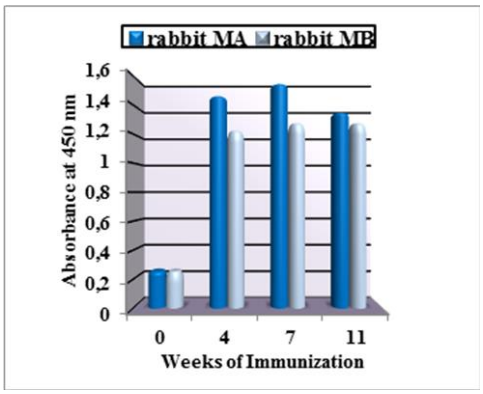


Fig. 5. ELISA result for the effect of duration of immunization.

After purification, the range of protein concentration of fractions collected which measured by Bradford Assay were 0.5-2 µg/ µl. The SDS-PAGE analysis result showed a single band of IgG about 150 KDa (Figure 6). ELISA results showed the titer of purified polyclonal anti-M2 IgG is (1:32000) (Fig. 7).

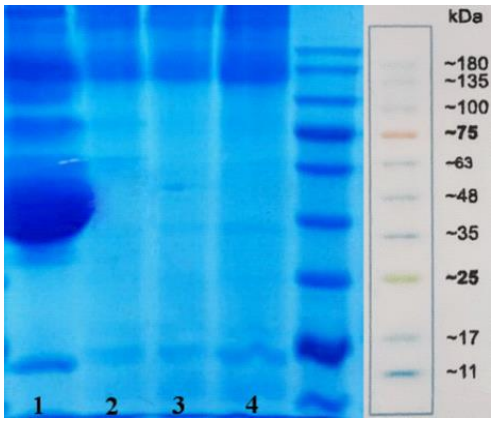


Fig. 6. SDS-PAGE of the purified IgG-M2: (lane1) Primary antiserum; (lanes 2, 3 and 4) Purified IgG; (lane 5) protein marker.

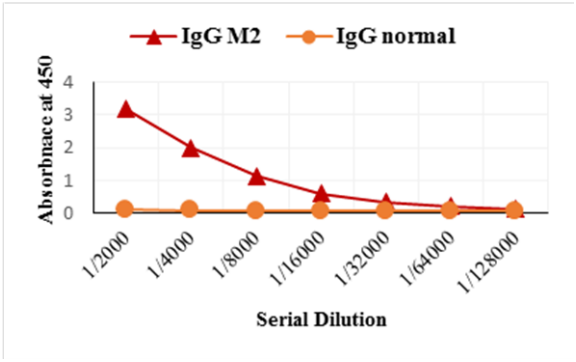


Fig. 7. Titer assay of the purified anti-M2 IgG and normal IgG.

Discussion

Polyclonal antibodies (pAb) are a collection of the antibodies which are secreted against epitopes of an antigen with different affinities. They can be obtained within a short period of time (4-8weeks). These antibodies are produced via injection of antigen including adjuvant to the laboratory animals such as rabbit. Adjuvants are often classified as immune stimulatory molecules or delivery systems. Antigen adsorption to adjuvant is important for immune response, thus, when selecting which adjuvant to be used, antigen hydropathicity is a critical consideration. Freund's adjuvant (CFA, IFA), one of the adjuvants which is preferably used for polyclonal antibody production in animals, is able to stimulate the immune system (10).

For years, polyclonal antibodies have been used in a wide range of clinical and research works in order to qualitative/quantitative analysis (ELISA and immunoblot), immuno-affinity purification, neutralization activity and immunotherapy(15). Production of low cost and simple techniques for IgG purification are advantages of using polyclonal antibodies in fundamental immunological research, diagnostic testing and vaccine quality control (16).

Considering the role of influenza virus in seasonal infections which lead to the death of about 250,000 people annually, the production of polyclonal antibodies against critical virus proteins such as M2 protein is necessary. Although, hemagglutinin is the most important viral antigen, M2 protein is a critical protein in cell entry and establishment of influenza infection.

Anti-M2pAb have different applications including their role in passive immunization due to their neutralization activity (4). Also, anti-M2pAb may show better results than M2 monoclonal antibody used for the treatment of the influenza virus severe infections (16).

The suitability of antibodies for different purposes is very important issue. Each antibody couldn't work equally well in all assays. An antibody for example may work good in a Western blot analysis but fail in immunohistochemistry tests. Nature of antibody in the epitopes recognition, depends on the epitope structure in the antigen molecule. If an epitope is on the surface of the protein and is made up by amino acids that are widely separated in the primary structure of the protein, then the denaturation of the protein on an SDS gel may irreversibly separate and disrupt this epitope. This consideration is an important approach to study the proteins using immunoassay techniques.

Immunochemical techniques use antibodies directed against conformational epitopes. They mostly include enzyme-linked immunosorbent assay (ELISA), immunonephelometry, SRID and Plasmon Surface Resonance. In this study we used 300µg of purified recombinant M2 protein mixed with Freund's adjuvant for each animal which is compatible to the usual dose (the range of 50 to 1000 µg) of soluble protein administered to rabbits.

We successfully expressed and purified the expressed recombinant M2 protein in E.coli as prokaryotic system. These steps are time-consuming, but are very important for specific antibody production. Because the level and specificity of the antibodies evoked by crude and purified antigens.

Furthermore, the specificity and suitability of this produced antibody was verified using immunological techniques such as Western blotting, ELISA and RID. In RID, the antigen-antibody complex is formed when either components (antibody and antigen) collided together in agarose gel and sedimentary line or zone was observed. According to the work of Jasem Estakhr et al in 2011, the formation of complex depends on compounds concentration and molecular weight. Also, based on antigen molecular weight, appropriate gel percentage should be used (17). The RID results in this study indicated the interaction between M2 antigen and M2 polyclonal antisera. In SRID, the diameter of sedimentary zones increased with increasing the M2 protein concentration. Furthermore, double zones were observed which is for antiserum and antigen homologous (18).

Also, the immunoblotting results of proteins contains protected domains especially M2e and the results of Table 2 shows that pAb can detect M2 protein epitopes upon other recombinant proteins of the influenza virus. Additionally, the data obtained from NCBI have shown that M2 native protein amino acid sequences have more than 97% homology in all influenza A virus strains (19). Accordingly, it can be predicted that anti-M2pAb is able to detect M2 proteins of all type A viruses.

Eventually, according to protein evaluation and comparison with data provided by the producer companies of the anti-M2pAb, IgG produced in this study is effective and has the potential to be used in clinical and research works.

Acknowledgements

This work was funded by grant No. 944, from Pasteur Institute of IRAN.

Ethical statements

According to license of the Iran Ethical Committee to No.IR.PII.REC.1395.82, we have permission experimental work with animals.

References

1. Price I, Mochan-Keef ED, Swigon D, Ermentrout GB, Lukens S, Toapanta FR, et al. The inflammatory response to influenza A virus (H1N1): An experimental and mathematical study. J Theor Biol. 2015;374:83-93.

2. Pielak RM, Chou JJ. Influenza M2 proton channels. Biochim Biophys Acta. 2011;1808(2): 522-9.

3. Gamblin SJ, Skehel JJ. Influenza hemagglutinin and neuraminidase membrane glycoproteins. J Biol Chem. 2010;285(37):28403-9.
4. Ye J, Shao H, Perez DR. Passive immune neutralization strategies for prevention and control of influenza A infections. Immunotherapy. 2012;4 (2):175-86.
5. Esfahani MAA, Fotouhi F, Ghaemi A, Saleh M, Chalabiani S, Kelishadi B, et al. Expression of M2 Protein of Human Influenza Virus in Escherichia Coli. J Isfahan Med Sch. 2013;30(216).
6. Meng Q, Liu G, Liu Y, Deng X, Wang W, Xu K, et al. A broad protection provided by matrix protein 2 (M2) of avian influenza virus. Vaccine. 2015;33(31):3758-65.
7. Stanekova Z, Vareckova E. Conserved epitopes of influenza A virus inducing protective immunity and their prospects for universal vaccine development. Virol J. 2010;7:351.
8. Esghaei M, Monavari SH, Tavassoti-Kheiri M, Shamsi-Shahrabadi M, Heydarchi B, Farahmand B, et al. Expression of the influenza M2 protein in three different eukaryotic cell lines. J Virological Methods. 2012;179(1):161-5.
9. Swinkels WJ, Hoeboer J, Sikkema R, Vervelde L, Koets AD. Vaccination induced antibodies to recombinant avian influenza A virus M2 protein or synthetic M2e peptide do not bind to the M2 protein on the virus or virus infected cells. Virol J. 2013;10:206.
10. Fox CB, Kramer RM, Barnes V L, Dowling QM, Vedvick TS. Working together: interactions between vaccine antigens and adjuvants. Ther Adv Vaccines. 2013;1(1):7-20.
11. Ponomarenko J, Bui H-H, Li W, Fusseder N, Bourne PE, Sette A, et al. ElliPro: a new structure-based tool for the prediction of antibody epitopes. BMC Bioinformatics. 2008;9(1):514.
12. Zamani S, Fotouhi Chahouki F, Nourmohammadi Z, Sadeghi Neshat S, Mazaheri V, Torabi A, et al. Production of polyclonal antibody against Tehran strain influenza virus (A/H1N1/2009) hemagglutinin conserved domain (HA2): brief report. Tehran Univ Med J. 2015;73 (7):535-9.
13. Ikai A. Thermostability and aliphatic index of globular proteins. J Biochem. 1980;88(6):1895-8.
14. Eichacker LA, Granvogl B, Mirus O, Müller BC, Miess C, Schleiff E. Hiding behind hydrophobicity transmembrane segments in mass spectrometry. J Biol Chem. 2004;279(49):50915-22.
15. Lipman NS, Jackson LR, Trudel LJ, Weis-Garcia F. Monoclonal versus polyclonal antibodies: distinguishing characteristics, applications, and information resources. ILAR J. 2005;46(3):258-68.
16. Kalenik B, Sawicka R, Góra-Sochacka A, Sirko A. Influenza prevention and treatment by passive immunization. Acta Biochim Pol. 2014;61(3):573-87.
17. Estakhr J, Javdan N. Antigenicity determination of purified ALPHA-TOXIN of clostridium septicum. Pharmacologyonline. 2011;2:835-41.
18. Vodeiko GM, Weir JP. Determination of H5N1 vaccine potency using reference antisera from heterologous strains of influenza. Influenza Other Respir Viruses. 2012;6(3):176-87.
19. Ebrahimi SM, Aghaiypour K, Nili H. Sequence analysis of M2 gene of avian influenza virus strain (A/Chicken/Iran/101/98 (H9N2)) as an oil vaccine seed. Iran J Biotechnol. 2008;6(4):235-138.