Journal of Poultry Sciences and Avian Diseases

Journal homepage: www.jpsad.com

Designing and Computational Analysis of Chimeric Avian Influenza Antigen: A Yeast-Displayed Universal and Cross-Protective Vaccine Candidate

Elyas Mohammadi[1], Mohammad Hadi Sekhavati[2*], Zana Pirkhezranian[2], Neda Shafizade[3], Samira Dashti[4] Naghmeh Saedi[5]

[1] Division of Neurogeriatrics, Department of Neurobiology, Care Sciences and Society, Karolinska Institutet, Stockholm, Sweden
[2] Department of Animal Science, Ferdowsi University of Mashhad, Mashhad, Iran
[3] Department of Internal Medicine, Mashhad University of Medical Science, Mashhad, Iran
[4] Department of Medical Science, Mashhad University of Medical Science, Mashhad, Iran
[5] Department of Animal science, Azad University of Tehran, Tehran, Iran

*** Corresponding author email address**: sekhavati@um.ac.ir

A r t i c l e I n f o

Article type:
Original Research

How to cite this article:
Mohammadi, E., Sekhavati, M. H., Pirkhezranian, Z., Shafizadeh, N., Dashti, S., Saedi, N., & Razmyar, J. (2023). Designing and Computational Analysis of Chimeric Avian Influenza Antigen: A Yeast-Displayed Universal and Cross-Protective Vaccine Candidate. *Journal of Poultry Sciences and Avian Diseases, 1*(1), 1-15.
http://dx.doi.org/10.61838/kman.jpsad.1.1.1

A B S T R A C T

This study describes the development of a cross-protective vaccine candidate against avian influenza virus, which was designed using M2e, a highly preserved antigen. The consensus sequence of M2e was obtained using 31 sequences of avian influenza virus subtypes (H5N8, H5N1, H9N2, and H7N9) isolated from seven avian species in five Asian countries. An adjuvant, a partial sequence of flagellin, was also considered. Two chimeric antigens were designed and virtually cloned and expressed using the PYD1 vector and EBY100 yeast strain. Molecular dynamic simulations were used to assess the stability and conformational features of these antigens. The likelihood of detection by a specific monoclonal antibody, MAb148, was estimated for the designed peptides using docking studies. The second chimeric antigen was more compact and stable than the first design, but it was less detectable by MAb148. In the first design, two of the four desired epitopes ("SLLTEVETP") were exposed, while only a partial sequence of this epitope was detectable in the second design. In contrast to the second chimeric antigen, electrostatic, and binding energies related to the interaction of the first antigen and MAb148 were significantly closer to the positive control. This suggests that epitopes of the first chimeric antigen could be correctly located in the specific paratope of MAb148. In conclusion, the first chimeric antigen exhibits favorable conformational features and epitope-paratope interactions, highlighting its potential as a promising cross-protective vaccine candidate against a range of avian influenza virus subtypes.

Keywords: *Bioinformatics, Avian influenza Virus, Chimeric Antigen, Monoclonal Antibody, Vaccine Candidate.*

Article history:
Received 02 December 2022
Accepted 23 February 2023
Revised 27 February 2023
Published online 01 March 2023

1 Introduction

Influenza virus consists of a single-stranded genomic fragment originated from the orthomyxoviridae family. Currently, this family is categorized into 5 types of influenza: A, B, C, Thogotovirus and Isavirus. Of these categories, only type A can be pathogenic in birds and it is classified into various subtypes with different levels of pathogenicity according to genetic variation in the surface glycoproteins of hemagglutinin (HA) (16 HA) and neuraminidase (NA) (9 NA) (1). Due to significant genetic diversity even between viruses of the same pandemic subtype, these influenza viruses encounter a much weaker immune response during initial infection. As a result, prevalence of pandemic influenza can cause disease, death and economic losses in the avian industry (2, 3). Commercially available antiviral drugs against influenza (4, 5) face challenges, as the virus can evolve and develop resistance, leading to reduced effectiveness or even ineffectiveness of treatment. Between 2008 and 2009, nearly 100% of seasonal influenza H1N1 and H3N2 subtypes in the United States were resistant to Oseltamivir and Zanamivir (two commercially available antiviral drugs), respectively.

Efforts to produce a recombinant vaccine using HA and NA as the two major antigens of influenza virus have encountered many problems due to genetic variability (4, 6). Vaccination with a conserve antigen that is resistant to mutations could confer immunity against several influenza subtypes (7-9). In this regard, the Matrix 2 (M2) may be the most promising antigen. The extracellular domain of this antigen, called M2e, is exposed and can be recognized by the immune system. This segment consists of 24 amino acids and forms an ion channel that plays a vital role in virus replication (7-10). Nine amino acids in this segment are remarkably conserved among all influenza subtypes (11, 12). This section is completely identical in 1,364 sequences extracted from the NCBI database (13). The “SLLTE” sequence which is the main core conferring antigenicity of M2e, has 97%, 98%, and 98% identity in human, swine and avian m2e sequences, respectively (13).

Despite the lower antigenicity of M2e peptide compared to HA and NA, it is considered a target for vaccine design due to its conserved sequence across all influenza A subtypes isolated since 1918 (14, 15). Moreover, the expression of this peptide on the surface of infected cells is two times higher than that of HA (10), which may compensate for its lower antigenicity (16). Using the M2e antigen alone may weakly provoke the immune system, but studies have shown that multiple copies of this peptide sequence increase immunogenicity (17-20). In vivo studies have revealed that M2e-specific antibodies can reduce lesions or mortality from infection with several influenza subtypes (21-23). In previous studies, fusing M2e to various adjuvants or carriers enhanced its antigenicity and provided protection against lethal challenges (24). Many investigations have also demonstrated that M2e-based vaccines can generate immunity against different influenza subtypes (11, 17, 19, 25-27). Additionally, M2e vaccines have been suggested as a supplement to increase the cross-protection of conventional vaccines (24, 28). A live attenuated influenza vaccine combined with M2e Virus-like particles (VLPs) protected mice against lethal challenges of H3N2, H1N1, and H5N1 (28).

Flagellin, the main component of bacterial flagella, is essential for bacterial movements. It is considered as one of the most potent stimulators of immune system, acting as a ligand that activates Toll-Like Receptor 5 (TLR5) on host cells (29-32). TLR5 Stimulation leads to the activation of the innate immune system (33). Chimeric proteins containing flagellin and a pathogen-specific antigen have been explored as vaccines against various infections including Neil, Malaria, Plague and Tuberculosis (34-37). Several studies have highlighted the potential of flagellin as an effective adjuvant (34). Other advantageous properties of flagellin as a vaccine component include: a) efficacy at low doses (38); b) lack of IgE response induction (39); c) the pre-existing immunity does not interfere with its adjuvant function (39, 40); d) no detected toxicity was detected in rabbits after nasal or intramuscular administration; and e) ease of large-scale production (33).

Recently, yeast-displayed vaccines for influenza have been investigated and shown potential benefits such as scalable production (41-45) without the need for external adjuvants like aluminum (42). Compared to soluble antigen expression, displaying antigens on the yeast cell surface enhances immunogenicity and immune recognition (45-49). In the light of the above, we designed a yeast-displayed chimeric avian influenza antigen comprising the M2e peptide and flagellin. Finally, computational approaches were used to predict the fidelity and immunogenicity of this chimeric antigen.

2 Methods and Materials

2.1 Determining the Consensus Sequence of M2e Antigen for Four Subtypes of Avian Influenza Virus

Influenza virus was determined based on a protocol developed by Huleatt et al. in 2008 (16) and Mozdzanowska et al. in 2003 (19). A total of 31 M2e protein sequences isolated from seven avian species originated from five Asian countries (China, Japan, India, South Korea and Vietnam) were retrieved from NCBI database. The CLC Workbench 5 software was used for the alignment of the sequences to obtain the consensus sequence of the antigen from H5N8, H5N1, H9N2 and H7N9 subtype of avian influenza (Table 1).

Table 1. 31 M2e Sequences of 4 Subtypes of Influenza from 7 Avian Species which was originated from 5 Asian countries.

subtype	Name	Accession number	Source	Country
H9N2	A/chicken/Beijing/1/1994(H9N2)	AAD49084.1	Chicken	China
	A/chicken/Guangxi/2389/2005(H9N2)	ABV47370.1		
	A/chicken/Jilin/A/2012(H9N2)	AHB73958.1		
	A/chicken/Henan/L1/2002(H9N2)	ACH68516.1		
	A/Chicken/Hong Kong/739/94(H9N2)	AAD49080.1		
	A/chicken/Shandong/244/2002(H9N2)	AHA38384.1		
	A/chicken/Yokohama/aq45/2002(H9N2)	BAF46480.1	Chicken	Japan
	A/chicken/Kobe/aq26/2001(H9N2)	BAF46450.1		
	A/chicken/Osaka/aq69/2001(H9N2)	BAF46470.1		
	A/chicken/India/01CL1826/2012(H9N2)	AKU37804.1	Chicken	India
	A/chicken/Korea/HC09/2009(H9N2)	ADQ43619.1	Chicken	South Korea
	A/chicken/Korea/SH0911/2009(H9N2)	ADQ43637.1		
H5N8	A/duck/Eastern China/S1109/2014(H5N8)	AJS16240.1	Duck	China
	A/duck/Eastern China/S1210/2013(H5N8)	AJS16222.1		
	A/duck/Shandong/Q1/2013(H5N8)	AIT38319.1		
	A/goose/Zhejiang/925037/2014(H5N8)	ALP30088.1	Goose	
	A/baikal teal/Korea/1449/2014(H5N8)	AIA61825.1	Baikal teal	South Korea
	A/environment/Korea/W487/2015(H5N8)	ANM71714.1	Environment	
H5N1	A/chicken/Gansu/6/2012(H5N1)	ANG09373.1	Chicken	China
	A/chicken/Jiangsu/18/2008(H5N1)	ADG59128.1		
	A/Duck/Hunan/191/05(H5N1)	AAZ16348.1	Duck	
	A/chicken/Jilin/hg/2002(H5N1)	ABI97330.1	Chicken	
	A/chicken/Viet Nam/TMU016/2008(H5N1)	AIY55071.1	Chicken	Vietnam
	A/duck/Viet Nam/Ncvd1/2003(H5N1)	ABP35636.1	Duck	
	A/whooper swan/Hokkaido/3/2011(H5N1)	BAL04278.1	Whooper swan	Japan
H7N9	A/wild bird/Korea/A14/2011(H7N9)(	AEK84655.1	Wild birds	South Korea
	A/wild duck/Korea/MHC39-13/2011(H7N9)	AGG53418.1	Wild Duck	
	A/duck/Shanghai/SD015/2015(H7N9)	ASV61924.1	Duck	China
	A/duck/Jiangxi/3214/2009(H7N9)	AGQ81228.1		
	A/duck/Jiangxi/3292/2009(H7N9)	AGQ81348.1		
	A/chicken/Guangdong/Q1/2016(H7N9)	ASB32487.1	Chicken	

2.2 *Design of the Chimeric Antigens*

To design chimeric antigens, previous studies (16, 19, 33, 50-52) and information related to PYD1 shuttle vector (http://n2t.net/addgene:73447) (53) and EBY100 yeast strain (genetically modified and contains the plasmid, pIU211 stably integrated into the genome for yeas-displaying proteins (48)) were used. To this end, after obtaining consensus sequence of M2e protein for H9N2, H5N1, H5N8 and H7N9 subtypes of avian influenza virus, a partial sequence of flagellin (retrieved from 5GY2, a TLR5-flagellin complex) was selected as an adjuvant to be fused to the consensus sequence of M2e (54). The AGA2 protein which is expressed by the PYD1 vector, was used to bind to the Aga1 protein on the surface of EBY100 yeast strain. A flexible linker (ASGGGGSGGGGSGGGGS) was considered twice, between AGA2 and the next protein and also between flagellin and four tandem copies of M2e antigen. Based on this information, two different chimeric proteins were designed for surface display on the EBY100 yeast strain (Figure 1).

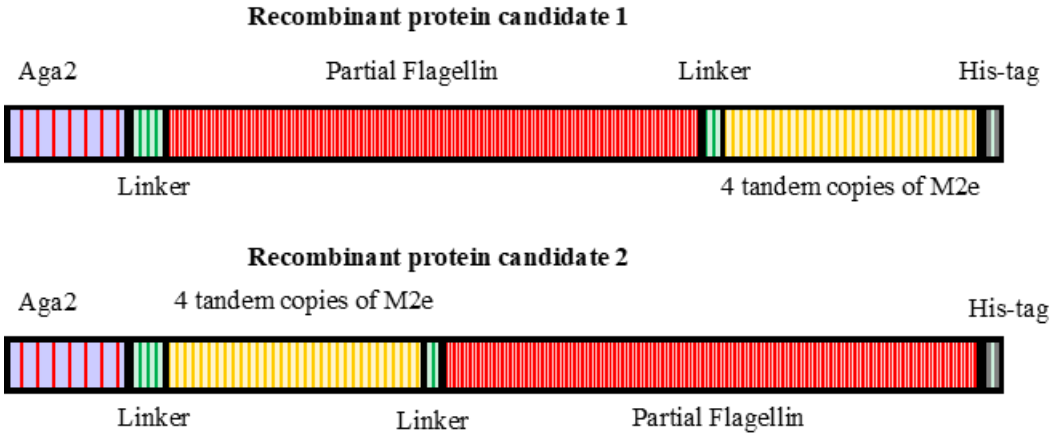

Figure 1. A schematic representation of two candidates of chimeric antigens for the H5N8, H5N1, H9N2 and H7N9 subtypes of avian influenza virus. AGA2: a protein that can be expressed by the PYD1 vector for binding to Aga1 protein on the surface of EBY100 yeast strain; flexible linker is ASGGGGSGGGGSGGGGS, the four tandem copies of M2e are (SLLTEVETPTRNGWECRCSDSSDPLV)4, partial sequence of flagellin is considered as an adjutant, and His-tag is for purification purposes.

2.3 *Protein Modeling*

To predict the function of designed chimeric antigens, their 3-dimensional structures were modeled using the I-TASSER server. In this regard, the structure of designed proteins was predicted through I-TASSER (55) server. In all four replications of the M2e protein, two cysteines were substituted with serine to prevent undesired disulfide bonds (SLLTEVETPTRNGWESRSSDSSDPLV) (16, 19). Modeling of the designed proteins was carried out using chain D of the 5GY2 crystallography structure. The loop structure of the M2e protein bound to the monoclonal antibody was applied to model the "SLLTEVETP" epitope.

2.4 *Molecular dynamic simulations*

Molecular dynamic simulations were used to predict the structure of the designed proteins under avian physiological conditions (avian normal body temperature and pressure). All simulations were carried out using GROMACS 5 program (56, 57). Two designed Proteins and docked complexes in further steps (protein-antibody) were processed under GROMOS 54a7 (58) force field library. Besides, SPC water model (59) was used for the solvation in a periodic cubic box that was large enough to contain the system and 1 nm of solvent on all sides. A combination of Na^+ and Cl^- were used to neutralize the solvated complex. Neutral systems were then subjected to the steepest descent energy minimization. After energy minimization each system was equilibrated for 200ps under NVT and NPT conditions. Temperature was set at 313K.The final production simulation was carried at NPT condition without any restraints. Pressure and temperature of the system were controlled by the Parrinello- Rahman (60) and V-rescale (61) algorithms respectively. The LINCS algorithm was used to constrain all the bond lengths (62). A Verlet cutoff method was used for no bonded interactions. No bonded interactions within 1 nm were updated every 20 steps. Trajectories were

analyzed with the help of VMD and Xmgrace (63). Using g_mmpbsa tool (64), van der Waals, electrostatic and binding energies for the interaction of designed proteins and complement-determining regions (CDR) of specific monoclonal antibody (in docking studies) were calculated. The same calculation was done for a positive control (5DLM, crystallography of naturalizing antibody and desired epitope) through g_mmpbsa tool for further assessments.

2.5 Docking studies

Before conducting docking studies, the 3D structure of the designed recombinant antigens which were prepared through protein modeling and MD simulations, were refined using ReFOLD server (65) to correct any residues in disallowed regions. The accuracy of the predicted models before and after refinement was evaluated using Ramachandran plot analysis. The refined models were then used to investigate their probability of binding to the CDR region of a specific monoclonal antibody (MAB148, retrieved from 5DLM complex). Docking studies were performed using the antibody mode of the Cluspro server (66). The position of the epitopes in the specific pocket of the antibody was subsequently assessed and visualized using Pymol 1.8 software (67).

3 Results

3.1 Determining the consensus sequence of M2e antigen

Based on the conservation of the M2e protein in different subtypes of avian influenza, only a few differences were observed within and between the four subtypes. As a result, the sequence "SLLTEVETPTRNGWECRCSDSSDPLV" was proposed as the consensus sequence of the M2e antigen for the H9N2, H5N1, H5N8 and H7N9 subtypes of avian influenza (Figure 2).

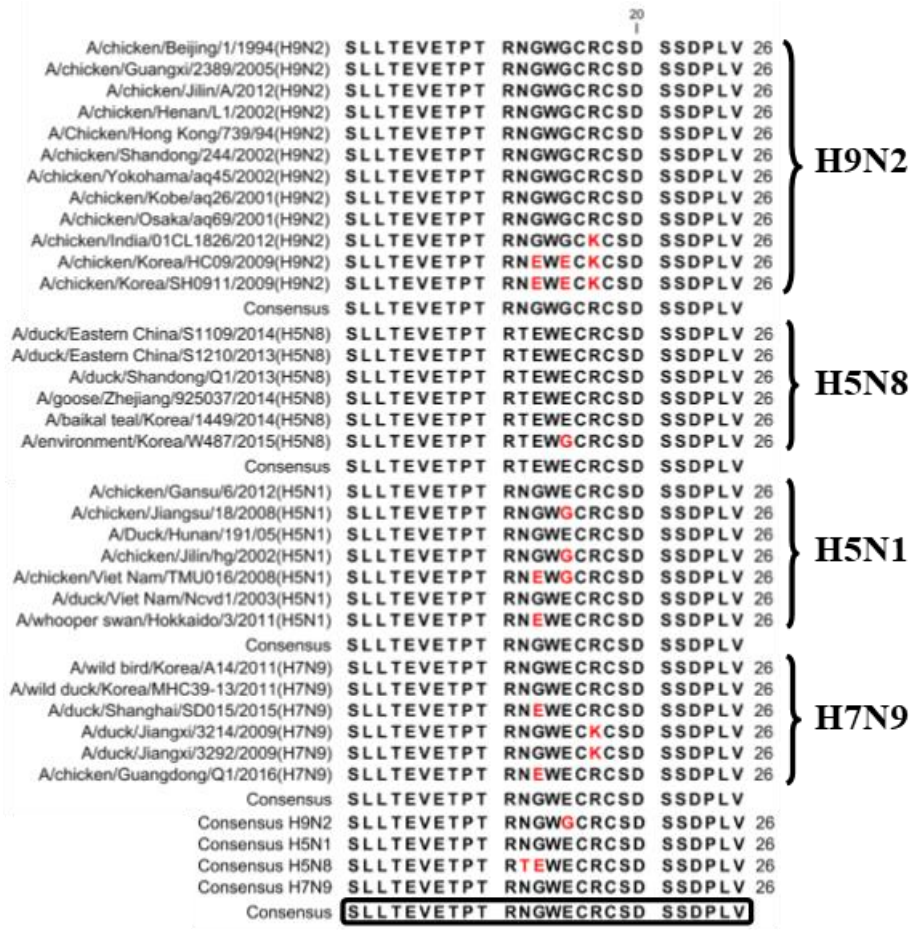

Figure 2. The consensus sequence of M2e protein for 4 subtypes (H9N9, H5N8, H5N1 and H9N2) of avian influenza virus were prepared from the sequences which belonged to 7 avian species and were isolated from Asian (China, Japan, India, South Korea and Vietnam) birds.

3.2 Protein modeling and dynamic simulations

After determining the consensus sequence of the M2e protein from four subtypes of avian influenza virus, the two designed proteins (Figure 1) were modeled using the I-Tasser server. The structural stability of recombinant proteins was investigated using GROMACS over a period of 100 nanosecond (ns). According to the RMSD plot (Figure 3, B), both recombinant proteins became stable after 20ns. Although there were several fluctuations in RMSD plot of both designs, the changes reported were less than 0.2 Angstrom. Regarding the candidate 1 antigen, the structural compactness of recombinant protein is less than that of second scheme (Figure 3, A). The flagellin region is completely distanced from other parts and the antigenic section lacks any space barrier for binding to specific antibodies. Additionally, AGA2 protein is free to bind to the yeast surface. On the other hand, the structural compactness of the candidate 2 antigen approximately caused inaccessibility to different sections, which may play a vital role in the stability of protein. However, this feature may also diminish the probability of antigenic recognition by specific antibodies, as comfited by docking analysis (Table 2).

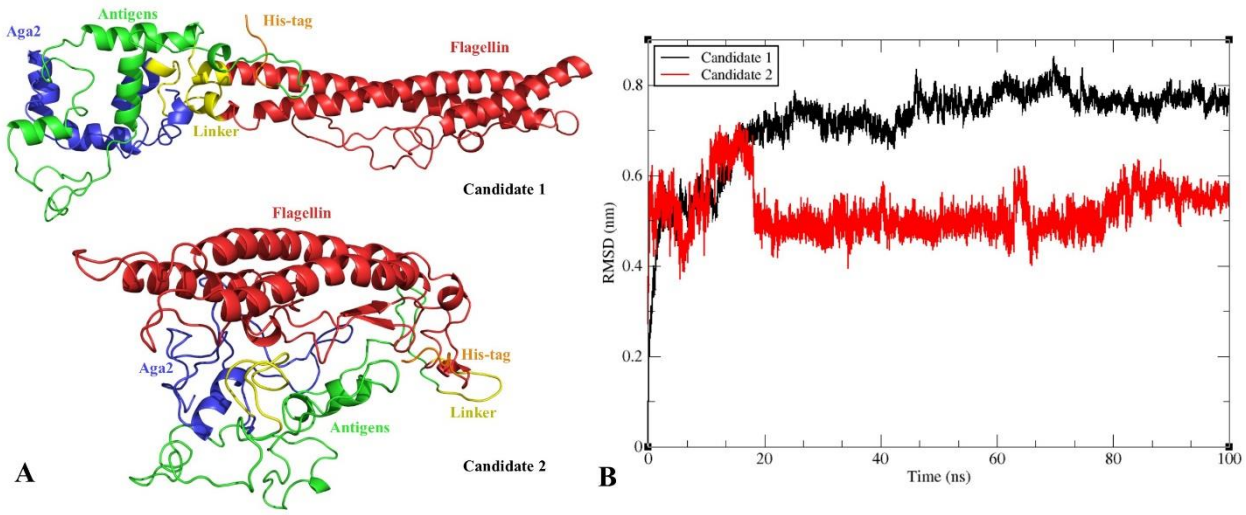

Figure 3. A) 3-Dimensional structure of 2 proposed designs as yeast-displayed candidates after 100 nanoseconds MD simulations B) The RMSD plot of both proteins based on alpha carbon space variation during the MD simulations.

3.3 Docking and binding energy

The Ramachandran plot analysis before and after structural refinement for the models of the chimeric antigens revealed significant changes for residues located in disallowed regions (Table 3). The results of docking studies for the two refined chimeric antigens revealed that two of the four desired epitopes ("SLLTEVETP") in the candidate 1 chimeric protein were fully recognizable by the CDR region of a specific monoclonal antibody. In contrast, only a section of one desired epitope was detectable in the other candidate chimeric protein. Based on the results obtained from the g_mmpbsa tool, even though there is slightly stronger Van der Waal energy between $_{143}$EVETPTRNG$_{151}$ epitope in candidate 2 chimeric protein and its specific antibody when compared to the two $_{329}$SLLTEVETP$_{337}$ and $_{277}$SLLTEVETP$_{285}$ epitopes in candidate 1, the electrostatic and binding energy of the identifiable epitopes of candidate 1 are significantly higher. Overall, the reported energies for epitopes of candidate 1 chimeric protein have values that are closest to the positive control which includes the binding, electrostatic, and Van der Waal energies between the "SLLTEVETP" epitope and the CDR region of a specific monoclonal antibody in a crystallographic structure (5DLM) (Table 2).

Table 2. Van der Waal, electrostatics and binding energies and also involved residues in the interaction of "SLLTEVETP" epitope in positive control, candidate 1, and candidate 2 chimeric protein with the CDR region of the specific monoclonal antibody. Positive control: The "SLLTEVETP" epitope and CDR region of specific monoclonal antibody in crystallography structure (ID: 5DLM). 1329-337 and 1277-285: First and second detectable epitope from candidate 1 chimeric protein by CDR region of specific monoclonal antibody (MAB148). 2143-151: the only detectable epitope from candidate 2 chimeric protein by CDR region of specific monoclonal antibody (MAB148).

	Position	van der Waal energy (kJ/mol)	Electrostatic energy (kJ/mol)	Binding energy (kJ/mol)
Positive control	SLLTEVETP	-302.5 +/- 1.516	-814.240 +/- 5.420	-606.778 +/- 1.940
$1_{329-337}$	$_{329}$SLLTEVETP$_{337}$	-193.096 +/- 1.308	-570.963 +/- 4.855	-429.755 +/- 2.265
$1_{277-285}$	$_{277}$SLLTEVETP$_{285}$	-188.554 +/- 0.988	-580.643 +/- 1.984	-374.152 +/- 2.96
$2_{143-151}$	$_{143}$EVETPTRNG$_{151}$	-200.803 +/- 1.924	-330.197 +/- 2.734	-246.158 +/- 2.408

Analysis of docking (residues which involve in interactions)										
Positive control	Antibody	Glu39	Ser52	Ser53	Gly54	Gly96	Tyr101	Gly101	Thr102	Ser103
	epitope	Ser2	Leu3	Leu4	Thr5	Val7	Glu8			
$1_{329-337}$	Antibody	Lys55	Tyr58	Tyr100	Tyr101					
	epitope	Ser329	Leu331	Thr332	Val334	Glu335				
$1_{277-285}$	Antibody	Glu39	Ser53	Gly54	Lys55					
	epitope	Ser277	Thr280	Glu283						
$2_{143-151}$	Antibody	Tyr37	Glu39	Ser52	Gly54	Tyr58	Tyr101			
	epitope	Glu145	Thr148	Arg149	Asn150	Gly151				

Table 3. Retrieved data from Ramachandran plot of designed proteins, prior to refinement and afterward.

Disallowed regions	Generously allowed regions	Additional allowed regions	Most favored regions	Model of designed proteins	
1.8%	0.3%	14.6%	83.3%	Candidate 1	Prior to refinement
0.6%	2.7%	17.6%	79.0%	Candidate 2	
1.2%	0.9%	11.6%	86.3%	Candidate 1	After refinement
0.3%	2.1%	10.9%	86.6%	Candidate 2	

With regard to the residue involvement in antibody-epitope docking results, the epitopes $1_{329-337}$ ($_{329}$SLLTEVETP$_{337}$) and $1_{277-285}$ ($_{277}$SLLTEVETP$_{285}$), which belong to the candidate 1 chimeric protein, were more similar to positive control (Table 2) (Figure 4).

According to the docking results, the location of the two desire epitopes of candidate 1 chimeric protein within the CDR region of a specific monoclonal antibody indicates that these epitopes are accurately positioned in the relevant pocket of the antibody (Figure 5). This finding suggests that humoral immunity can be accurately and specifically boosted.

3.4 Virtual cloning

Based on the results of docking studies and molecular dynamic simulations, as well as the structural properties of the two candidate proteins, it can be concluded that protein candidate 1 chimeric protein is a better choice for expression on the yeast surface. Therefore, the coding sequence of this protein can be selected for expression on the surface of Saccharomyces cerevisiae (EBY100). By flanking the coding sequence of candidate 1 chimeric protein between NheI at the beginning and XhoI at the end, this sequence could be cloned into the PYD1 plasmid as an expression vector in Saccharomyces cerevisiae (EBY100). The virtual cloning of this chimeric sequence was performed and the results are depicted in Figure 6.

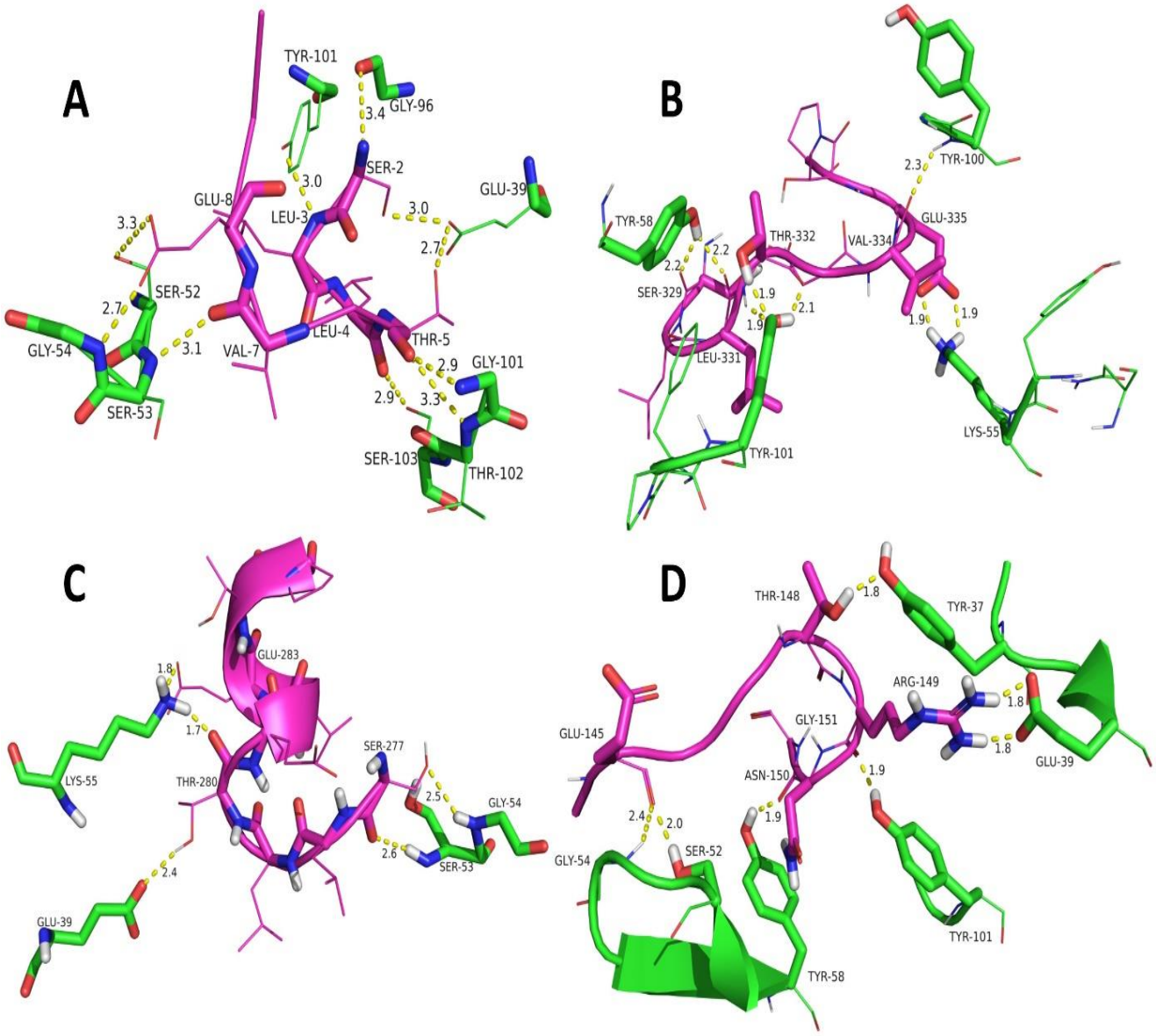

Figure 4. Cartoon representation of involved residues in docking of monoclonal antibody (green) and M2e protein epitopes (pink). A) Crystallography complex of monoclonal antibody and epitope (positive control) B) 1329-337 epitope and specific monoclonal antibody C) 1277-285 epitope and specific monoclonal antibody D) 2143-151 epitope and specific monoclonal antibody.

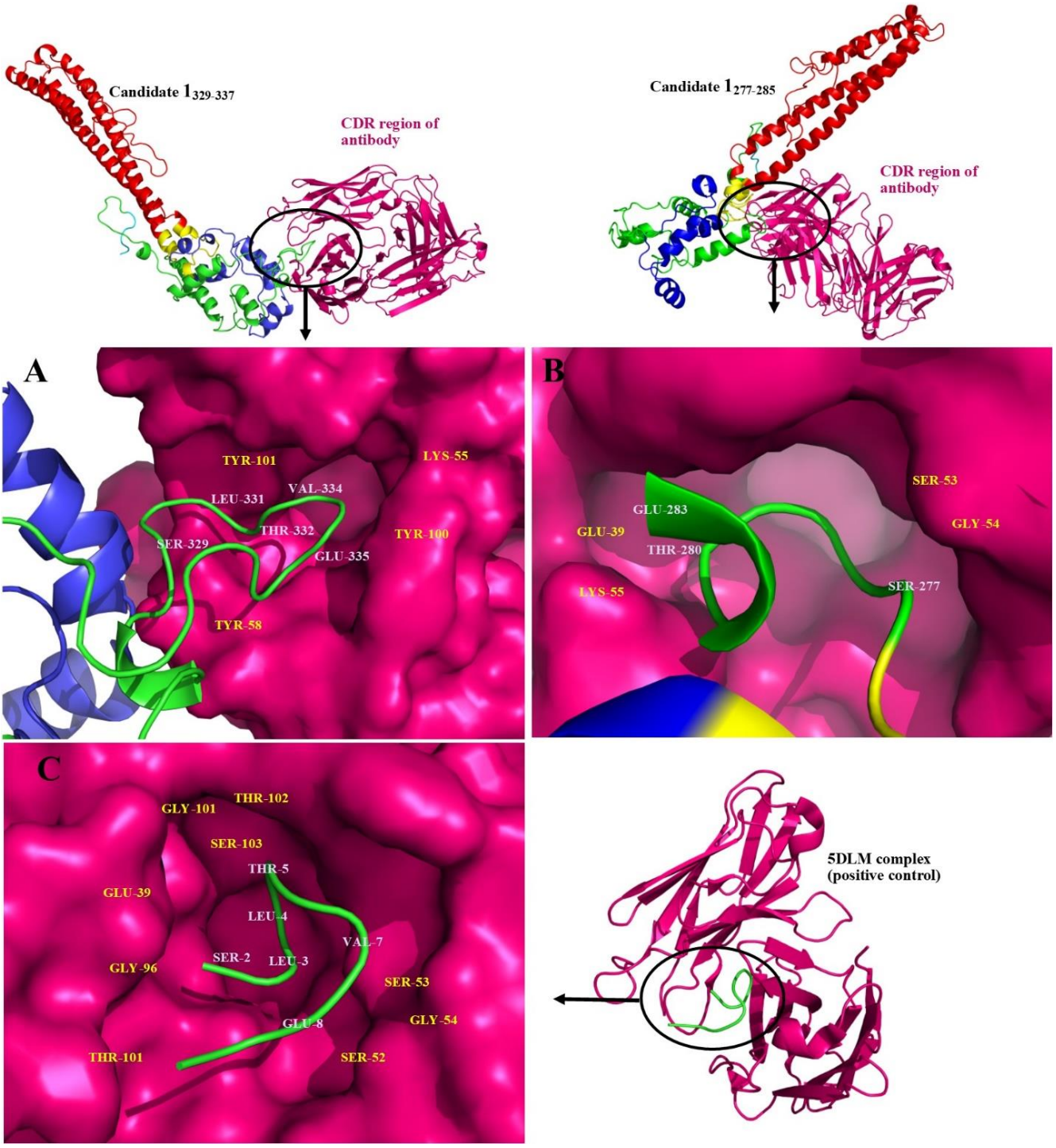

Figure 5. The position of 1329-337 and 1277-285 epitopes (candidate 1 chimeric protein) and "SLLTEVETP" epitope of crystallography (ID: 5DLM) in the specific pocket of CDR region of monoclonal antibody (the residues of antibody and epitope which involved in interactions are shown in yellow and white respectively). A) epitope 1329-337 B) epitope 1277-285. C) 5DLM complex (positive control).

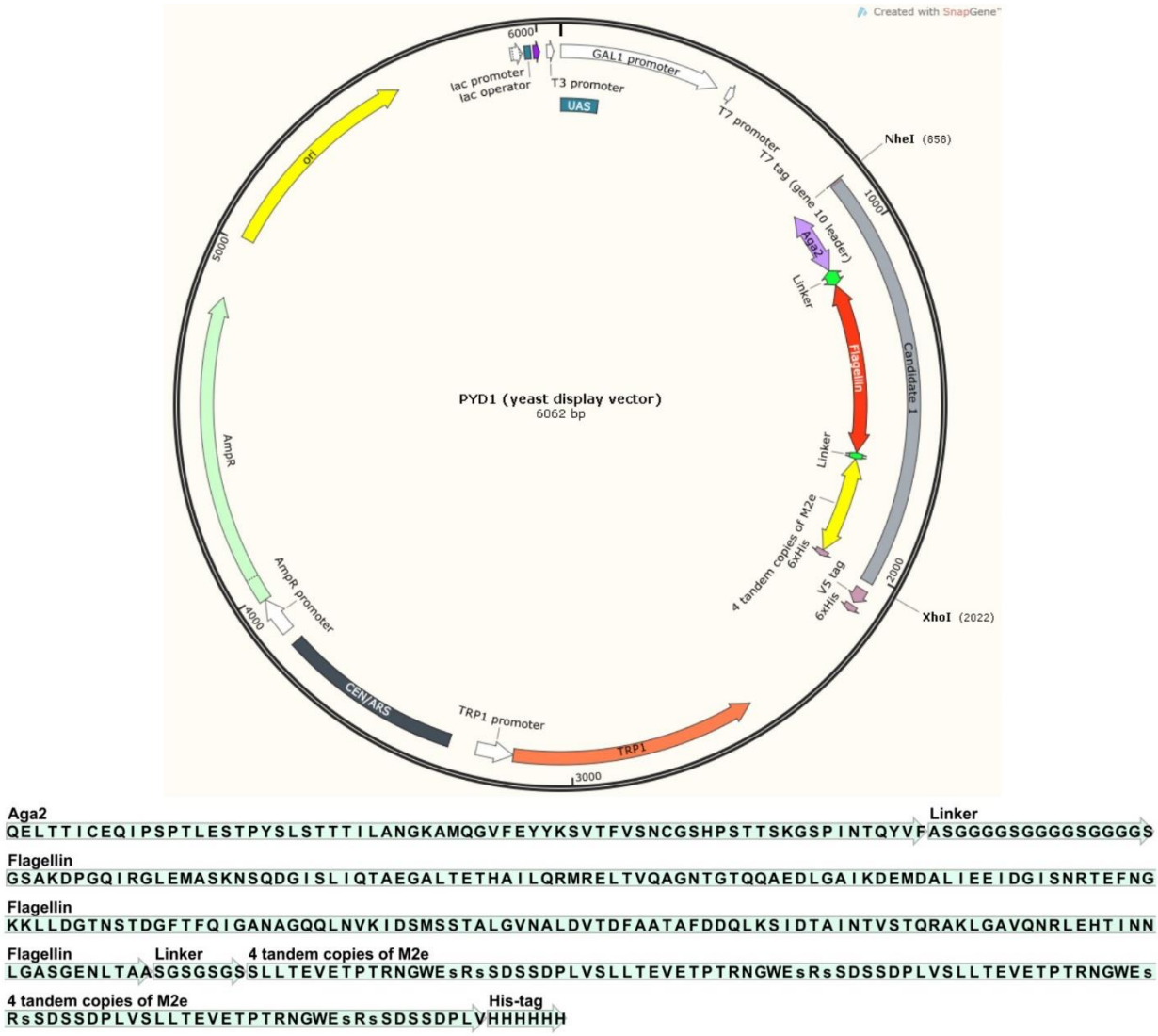

Figure 6. Virtual cloning of candidate 1 chimeric protein in PYD1 expression vector

4 Discussion

The pharmaceutical industry has been a significant and respected player since 19th century when it first began producing life-saving drugs like penicillin. Over time, the industry has developed many advancements, including the creation of vaccines, which has further cemented its reputation as a life-saving industry held in high esteem. However, in recent years, the industry's image has been tarnished due to the high cost of drug discovery, product recalls, adverse side effects, and increased consumer awareness and education. As a result, it is imperative that the industry finds ways to reduce the cost and time of drug discovery and increase drug target specificity to minimize side effects. Bioinformatics is one such tool that the industry has recently employed to aid in the drug discovery process while also reducing costs and timelines (68, 69). As a result, in this study we aimed to design and analyze the probability of producing a universal and cross-protective avian influenza vaccine through bioinformatics tools to reduce unnecessary laboratory costs.

In order to produce potential vaccines for various subtypes of influenza many efforts have been done so far (70). These efforts have failed due to high mutation rate of HA and NA as two major antigens of influenza virus (6). It seems to be rational to consider a preserved antigen against mutation to produce a cross-protective vaccine against various subtypes of influenza. M2e protein is one of the substantial antigens in this field. M2e peptide sequence has remained remarkably unchanged in influenza type A isolated since 1918 (14) and this feature made it an appropriate target for vaccine design. Based on previous studies invoked

antibodies against the epitopes of M2e antigen have diminished the growth of influenza virus in in vivo and in vitro studies and created cross-reactive resistance to influenza A subtypes. Based on a study conducted by Huleatt et al. in 2008 (16), among 7 sequences of M2e which was retrieved from 4 subtypes of influenza virus (H1N1, H2N2, H3N1, H3N2) a consensus sequence was obtained to be expressed in E. coli. Subsequently, mice immunized with this recombinant protein in aqueous buffer, without adjuvants or other formulation additives, developed potent M2e-specific antibody response. According to importance of avian influenza disease in China, Japan, South Korea, Vietnam and India, 31 M2e protein sequences for H9N2 (12 sequences), H5N8 (6 sequences), H5N1 (7 sequences) and H7N9 (6 sequences) subtypes were extracted from NCBI database. The selected subtypes of influenza are the most important and the most damaging influenza subtypes in these countries. H9N2 inflicts widespread damage in Iran and Iraq every year (68).

In previous studies, a recombinant protein comprising the TLR5 ligand flagellin fused to four tandem copies of the ectodomain of the conserved influenza matrix protein M2 (M2e, to overcome the low antigenicity of M2e in comparison with HA and NA) was expressed in E. Coli and purified to homogeneity. This protein, retained TLR5 activity and displayed the protective epitope of M2e defined by a monoclonal antibody, 14C2 (16). According to purification costs and toxicity of components of E. coli, the utilization of a better choice expression host seems to be rational. Recently, yeast-based vaccines have been investigated for influenza vaccination. Yeast as an expression host can facilitate and escalate the production of newly engineered antigens (41-45). Yeast-based vaccines do not require adjuvant (like aluminum) to stimulate the immune system (42).

First time, Li and colleagues in 2016 (52) indicated that the yeast which express H5N1 Hemagglutinin at its surface can be used as an influenza vaccine. The reason of choosing yeast is its ability to perform post translational processes and capability of stimulating immune system. This feature effectively activates dendritic cells and cytotoxic T cells (41). The recombinant yeast cells, simultaneously stimulate humoral and cellular immunity by presenting antigens to MHCI and MHCII pathways (42). Similarly to previous studies (16) we used 4 tandem copies of the obtained consensus sequences of the M2e protein and a partial sequence of flagellin as an adjuvant in our in-silico investigation, with the aim of expressing them on the surface of yeast. Two different structures were designed and their performance and features were evaluated through various procedures. According to docking studies, candidate 1 chimeric protein effectively exposes its epitopes to a specific monoclonal antibody (MAB148). The complement-determining regions (CDRs) of MAB148 form a deep and narrow binding pocket that accommodates the N-Terminal part of M2e. M2ePro10 and Ile11 emerge from the MAb148 binding pocket, where Pro10 kinks the M2e peptide such that its C-terminal segment is projected away from the monoclonal antibody (51). The two out of four epitopes of candidate 1 chimeric protein resemble a fishing hook, with residues Ser2-Leu3-Leu4-Thr5-Glu6 forming a β-turn that is complementary in shape to the MAb148 paratope.

As reported by Salleh et al. in 2012 (71), increasing the compactness of a protein can lead to an increase in its stability. However, despite the higher stability of candidate 2 chimeric protein, the epitopes of the M2e antigen in this protein are covered by other sections and are not detectable by the CDR region of the monoclonal antibody. Therefore, the compactness of a protein may have both benefits and drawbacks simultaneously. It is predictable that despite greater stability of candidate 2 chimeric protein compared to candidate 1, this protein may be able to elicit a weaker immune response than candidate 1. As per the molecular dynamic studies, Van der Waal, electrostatic and binding energies of the “SLLTEVETP” epitope to the CDR region of MAb148 monoclonal antibody in the first design (which is the most important epitope of M2e protein) were significantly higher than those in the second design. (11). Furthermore, the figures obtained from the first design were significantly closer to the positive control (i.e., the crystallographic structure between the "SLLTEVETP" epitope and MAB148 antibody) compared to candidate 2. As a result, unlike scheme 2, first design may be able to eliciting a stronger humoral immune response.

The significance of recognizing the “SLLTEVETP” epitope has been established in previous studies. Cho et al. (2016) (51) demonstrated that serine 2, leucine 3, leucine 4, and threonine 5 are crucial for binding to the monoclonal antibody MAB148 of the M2e protein. In contrast, Grandea et al. (2010) (13) demonstrated that serine 2, threonine 5 and glutamic acid 6 are essential for binding to TCN-031 and TCN-032 monoclonal antibodies. They expressed that these antibodies can recognize a core in “SLLTE” section of N-Terminal region of M2e protein, which comprises amino acids 2 to 6. Consequently, scheme 1 is expected to provide humoral immunity by MAB148, TCN-031 and TCN-032

antibodies. Due to the two exposed repetition of "SLLTEVETP" epitope in scheme 1, there is a significant possibility of producing three different specific monoclonal antibodies against this region.

The PYD1 shuttle vector and EBY100 yeast strain can be utilized to display the candidate 1 chimeric protein on the surface of yeast. The pYD1 is a 5.0 kb expression vector designed for expression, secretion, and display of proteins on the extracellular surface of recombinant Saccharomyces cerevisiae cells (EBY100). Features of this vector allow regulated expression, secretion, and detection of expressed proteins on the cell surface of EBY100 (53). The vector contains AGA2 gene from Saccharomyces cerevisiae. This gene encodes one of the subunits of the a-agglutinin receptor. Fusion of the gene of interest to AGA2 allows secretion and display of the protein of interest. EBY100 expresses the AGA1 gene under control of the GAL1 promoter (47) and the attachment of AGA2 and AGA1 lead to yeast-displaying a recombinant protein. In conclusion, the findings of this study suggest a promising chimeric antigen for avian influenza virus, which could serve as a universal and cross-protective avian influenza vaccine candidate or as a complement of conventional avian influenza vaccines as well. Our laboratory has already begun further research in this direction.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgments

The authors thank the colleagues in our consortium who collaborated greatly in the research and who contributed their knowledge and experience. Special thanks to Dr. Ali Javadmanesh and Dr. Tahmorespoor who generously helped us in the research.

Author Contributions

Conceptualization: M. H. Sekhavati, J Razmyar; Data curation: E. Mohammadi, Z. Pirkhezranian; Formal analysis: S. Dashty, N Saedi; Methodology: M. H. Sekhavati; Project administration:N. Shafizade, J. Razmyar; Writing – original draft: E. Mohammadi; Writing – review & editing: M. H. Sekhavati, J. Razmyar. All authors have read and agreed to the published version of the manuscript.

Data Availability Statement

Data are available from the first author upon reasonable request.

References

1. Alexander DJ. An overview of the epidemiology of avian influenza. Vaccine. 2007;25(30):5637-44. [PMID: 17126960]. https://doi.org/10.1016/j.vaccine.2006.10.051.
2. Carrat F, Flahault A. Influenza vaccine: the challenge of antigenic drift. Vaccine. 2007;25(39-40):6852-62. [PMID: 17719149]. https://doi.org/10.1016/j.vaccine.2007.07.027.
3. Barnes B, Scott A, Hernandez-Jover M, Toribio J-A, Moloney B, Glass K. Modelling high pathogenic avian influenza outbreaks in the commercial poultry industry. Theoretical Population Biology. 2019;126:59-71.
4. Gubareva LV, Kaiser L, Hayden FG. Influenza virus neuraminidase inhibitors. Lancet. 2000;355(9206):827-35. [PMID: 10711940]. https://doi.org/10.1016/s0140-6736(99)11433-8.
5. Wang C, Takeuchi K, Pinto LH, Lamb RA. Ion channel activity of influenza A virus M2 protein: Characterization of the amantadine block1993. 5585-94 p. PMID: 7688826]. [PMCID: PMC237962] https://doi.org/10.1128/jvi.67.9.5585-5594.1993.
6. Webster RG, Bean WJ, Gorman OT, Chambers TM, Kawaoka Y. Evolution and ecology of influenza A viruses. Microbiol Rev. 1992;56(1):152-79. .[PMID: 1579108] [PMCID: PMC372859] https://doi.org/10.1128/mr.56.1.152-179.1992.
7. Sugrue RJ, Bahadur G, Zambon MC, Hall-Smith M, Douglas AR, Hay AJ. Specific structural alteration of the influenza haemagglutinin by amantadine. Embo j. 1990;9(11):3469-76. [PMID: 2209554] [PMCID: PMC552095]. https://doi.org/10.1002/j.1460-2075.1990.tb07555.x.
8. Sugrue RJ, Hay AJ. Structural characteristics of the M2 protein of influenza A viruses: evidence that it forms a tetrameric channel. Virology. 1991;180(2):617-24. [PMID: 1989386] [PMCID: PMC7131614]. https://doi.org/10.1016/0042-6822(91)90075-m.
9. Holsinger LJ, Shaughnessy MA, Micko A, Pinto LH, Lamb RA. Analysis of the posttranslational modifications of the influenza virus M2 protein. J Virol. 1995;69(2):1219-25. [PMID: 7529332] [PMCID: PMC188695]. https://doi.org/10.1128/jvi.69.2.1219-1225.1995.
10. Lamb RA, Zebedee SL, Richardson CD. Influenza virus M2 protein is an integral membrane protein expressed on the infected-cell surface. Cell. 1985;40(3):627-33. [PMID: 3882238]. https://doi.org/10.1016/0092-8674(85)90211-9.
11. Fu TM, Freed DC, Horton MS, Fan J, Citron MP, Joyce JG, et al. Characterizations of four monoclonal antibodies against M2 protein ectodomain of influenza A virus. Virology. 2009;385(1):218-26. [PMID: 19070878]. https://doi.org/10.1016/j.virol.2008.11.035.
12. Wang R, Song A, Levin J, Dennis D, Zhang NJ, Yoshida H, et al. Therapeutic potential of a fully human monoclonal antibody against influenza A virus M2 protein. Antiviral Res. 2008;80(2):168-77. [PMID: 18598723]. https://doi.org/10.1016/j.antiviral.2008.06.002.
13. Grandea AG, 3rd, Olsen OA, Cox TC, Renshaw M, Hammond PW, Chan-Hui PY, et al. Human antibodies reveal a protective epitope that is highly conserved among human and nonhuman influenza A viruses. Proc Natl Acad Sci U S A. 2010;107(28):12658-63. [PMID: 20615945] [PMCID: PMC2906546] https://doi.org/10.1073/pnas.0911806107.

14. Reid AH, Fanning TG, Janczewski TA, McCall S, Taubenberger JK. Characterization of the 1918 "Spanish" influenza virus matrix gene segment. J Virol. 2002;76(21):10717-23. [PMCID: PMC136643]. https://doi.org/10.1128/jvi.76.21.10717-10723.2002.
15. Mezhenskaya D, Isakova-Sivak I, Rudenko L. M2e-based universal influenza vaccines: a historical overview and new approaches to development. Journal of biomedical science. 2019;26(1):76. [PMCID: PMC6800501]. https://doi.org/10.1186/s12929-019-0572-3
16. Huleatt JW, Nakaar V, Desai P, Huang Y, Hewitt D, Jacobs A, et al. Potent immunogenicity and efficacy of a universal influenza vaccine candidate comprising a recombinant fusion protein linking influenza M2e to the TLR5 ligand flagellin. Vaccine. 2008;26(2):201-14. [PMID: 18063235]. https://doi.org/10.1016/j.vaccine.2007.10.062.
17. Fan J, Liang X, Horton MS, Perry HC, Citron MP, Heidecker GJ, et al. Preclinical study of influenza virus A M2 peptide conjugate vaccines in mice, ferrets, and rhesus monkeys. Vaccine. 2004;22(23-24):2993-3003. [PMID: 15297047]. https://doi.org/10.1016/j.vaccine.2004.02.021.
18. Liu W, Peng Z, Liu Z, Lu Y, Ding J, Chen YH. High epitope density in a single recombinant protein molecule of the extracellular domain of influenza A virus M2 protein significantly enhances protective immunity. Vaccine. 2004;23(3):366-71. [PMID: 15530682]. https://doi.org/10.1016/j.vaccine.2004.05.028.
19. Mozdzanowska K, Feng J, Eid M, Kragol G, Cudic M, Otvos L, Jr., Gerhard W. Induction of influenza type A virus-specific resistance by immunization of mice with a synthetic multiple antigenic peptide vaccine that contains ectodomains of matrix protein 2. Vaccine. 2003;21(19-20):2616-26. [PMID: 12744898]. https://doi.org/10.1016/s0264-410x(03)00040-9.
20. De Filette M, Min Jou W, Birkett A, Lyons K, Schultz B, Tonkyro A, et al. Universal influenza A vaccine: optimization of M2-based constructs. Virology. 2005;337(1):149-61. [PMID: 15914228]. https://doi.org/10.1016/j.virol.2005.04.004.
21. Roberts PC, Lamb RA, Compans RW. The M1 and M2 proteins of influenza A virus are important determinants in filamentous particle formation. Virology. 1998;240(1):127-37. [PMID: 9448697]. https://doi.org/10.1006/viro.1997.8916.
22. Hughey PG, Roberts PC, Holsinger LJ, Zebedee SL, Lamb RA, Compans RW. Effects of antibody to the influenza A virus M2 protein on M2 surface expression and virus assembly. Virology. 1995;212(2):411-21. [PMID: 7571410]. https://doi.org/10.1006/viro.1995.1498.
23. Zebedee SL, Lamb RA. Influenza A virus M2 protein: monoclonal antibody restriction of virus growth and detection of M2 in virions. J Virol. 1988;62(8):2762-72. [PMID: 2455818].[PMCID: PMC253710]. https://doi.org/10.1128/jvi.62.8.2762-2772.198822.
24. Kang S-M, Kim M-C, W Compans R. Virus-like particles as universal influenza vaccines2014. [PMID: 23002980].[PMCID: PMC3513402]. https://doi.org/10.1586/erv.12.70.
25. Slepushkin VA, Katz JM, Black RA, Gamble WC, Rota PA, Cox NJ. Protection of mice against influenza A virus challenge by vaccination with baculovirus-expressed M2 protein. Vaccine. 1995;13(15):1399-402. [PMID: 8578816]. https://doi.org/10.1016/0264-410x(95)92777-y.
26. Neirynck S, Deroo T, Saelens X, Vanlandschoot P, Jou WM, Fiers W. A universal influenza A vaccine based on the extracellular domain of the M2 protein. Nat Med. 1999;5(10):1157-63. [PMID: 10502819]. https://doi.org/10.1038/13484.
27. Tompkins SM, Zhao ZS, Lo CY, Misplon JA, Liu T, Ye Z, et al. Matrix protein 2 vaccination and protection against influenza viruses, including subtype H5N1. Emerg Infect Dis. 2007;13(3):426-35. [PMID: 17552096] [PMCID: PMC2725899]. https://doi.org/10.3201/eid1303.061125.
28. Song JM, Wang BZ, Park KM, Van Rooijen N, Quan FS, Kim MC, et al. Influenza virus-like particles containing M2 induce broadly cross protective immunity. PLoS One. 2011;6(1):e14538. [PMID: 21267073] [PMCID: PMC3022578]. https://doi.org/10.1371/journal.pone.0014538.
29. Hase CC. Analysis of the role of flagellar activity in virulence gene expression in Vibrio cholerae. Microbiology. 2001;147(Pt 4):831-7. [PMID: 11283279]. https://doi.org/10.1099/00221287-147-4-831.
30. Hayashi F, Smith KD, Ozinsky A, Hawn TR, Yi EC, Goodlett DR, et al. The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. Nature. 2001;410(6832):1099-103. [PMID: 11323673]. https://doi.org/10.1038/35074106.
31. Gewirtz AT, Navas TA, Lyons S, Godowski PJ, Madara JL. Cutting edge: bacterial flagellin activates basolaterally expressed TLR5 to induce epithelial proinflammatory gene expression. J Immunol. 2001;167(4):1882-5. [PMID: 11489966]. https://doi.org/10.4049/jimmunol.167.4.1882.
32. Mizel SB, Snipes JA. Gram-negative flagellin-induced self-tolerance is associated with a block in interleukin-1 receptor-associated kinase release from toll-like receptor 5. J Biol Chem. 2002;277(25):22414-20. [PMID: 11953430]. https://doi.org/10.1074/jbc.m201762200.
33. Mizel SB, Bates JT. Flagellin as an adjuvant: cellular mechanisms and potential. J Immunol. 2010;185(10):5677-82. [PMID: 21048152] [PMCID: PMC3756556]. https://doi.org/10.4049/jimmunol.1002156.
34. Liu G, Song L, Reiserova L, Trivedi U, Li H, Liu X, et al. Flagellin-HA vaccines protect ferrets and mice against H5N1 highly pathogenic avian influenza virus (HPAIV) infections. Vaccine. 2012;30(48):6833-8. [PMID: 23000130]. https://doi.org/10.1016/j.vaccine.2012.09.013.
35. Bargieri DY, Rosa DS, Braga CJ, Carvalho BO, Costa FT, Espindola NM, et al. New malaria vaccine candidates based on the Plasmodium vivax Merozoite Surface Protein-1 and the TLR-5 agonist Salmonella Typhimurium FliC flagellin. Vaccine. 2008;26(48):6132-42. [PMID: 18804504]. https://doi.org/10.1016/j.vaccine.2008.08.070.
36. McDonald WF, Huleatt JW, Foellmer HG, Hewitt D, Tang J, Desai P, et al. A West Nile virus recombinant protein vaccine that coactivates innate and adaptive immunity. J Infect Dis. 2007;195(11):1607-17. [PMID: 17471430]. https://doi.org/10.1086/517613.
37. Mizel SB, Graff AH, Sriranganathan N, Ervin S, Lees CJ, Lively MO, et al. Flagellin-F1-V fusion protein is an effective plague vaccine in mice and two species of nonhuman primates. Clin Vaccine Immunol. 2009;16(1):21-8. [PMID: 18987167] [PMCID: PMC2620661].
38. Weimer ET, Ervin SE, Wozniak DJ, Mizel SB. Immunization of young African green monkeys with OprF epitope 8-OprI-type A- and B-flagellin fusion proteins promotes the production of protective antibodies against nonmucoid Pseudomonas aeruginosa. Vaccine. 2009;27(48):6762-9. [PMID: 19744586]. https://doi.org/10.1016/j.vaccine.2009.08.080.
39. Honko AN, Sriranganathan N, Lees CJ, Mizel SB. Flagellin is an effective adjuvant for immunization against lethal respiratory challenge with Yersinia pestis. Infect Immun. 2006;74(2):1113-20. [PMID: 16428759] [PMCID: PMC1360354]. https://doi.org/10.1128/iai.74.2.1113-1120.2006.

40. Ben-Yedidia T, Abel L, Arnon R, Globerson A. Efficacy of anti-influenza peptide vaccine in aged mice. Mech Ageing Dev. 1998;104(1):11-23. [PMID: 9751429]. https://doi.org/10.1016/s0047-6374(98)00045-1.
41. Saelens X, Vanlandschoot P, Martinet W, Maras M, Neirynck S, Contreras R, et al. Protection of mice against a lethal influenza virus challenge after immunization with yeast-derived secreted influenza virus hemagglutinin. Eur J Biochem. 1999;260(1):166-75. [PMID: 10091596]. https://doi.org/10.1046/j.1432-1327.1999.00150.x.
42. Stubbs AC, Martin KS, Coeshott C, Skaates SV, Kuritzkes DR, Bellgrau D, et al. Whole recombinant yeast vaccine activates dendritic cells and elicits protective cell-mediated immunity. Nat Med. 2001;7(5):625-9. [PMID: 11329066]. https://doi.org/10.1038/87974.
43. Tamaru Y, Ohtsuka M, Kato K, Manabe S, Kuroda K, Sanada M, Ueda M. Application of the arming system for the expression of the 380R antigen from red sea bream iridovirus (RSIV) on the surface of yeast cells: a first step for the development of an oral vaccine. Biotechnol Prog. 2006;22(4):949-53. [PMID: 16889368]. https://doi.org/10.1021/bp060130x.
44. Shibasaki S, Aoki W, Nomura T, Miyoshi A, Tafuku S, Sewaki T, Ueda M. An oral vaccine against candidiasis generated by a yeast molecular display system. Pathog Dis. 2013;69(3):262-8. [PMID: 23873745]. https://doi.org/10.1111/2049-632x.12068.
45. Shibasaki S, Ueda M, Ye K, Shimizu K, Kamasawa N, Osumi M, Tanaka A. Creation of cell surface-engineered yeast that display different fluorescent proteins in response to the glucose concentration. Appl Microbiol Biotechnol. 2001;57(4):528-33. [PMID: 11762600]. https://doi.org/10.1007/s002530100767.
46. Ye K, Shibasaki S, Ueda M, Murai T, Kamasawa N, Osumi M, et al. Construction of an engineered yeast with glucose-inducible emission of green fluorescence from the cell surface. Appl Microbiol Biotechnol. 2000;54(1):90-6. [PMID: 10952010]. https://doi.org/10.1007/s002539900307.
47. Boder ET, Wittrup KD. Yeast surface display for directed evolution of protein expression, affinity, and stability. Methods Enzymol. 2000;328:430-44. [PMID: 9181578]. https://doi.org/10.1038/nbt0697-553.
48. Boder ET, Wittrup KD. Yeast surface display for screening combinatorial polypeptide libraries. Nat Biotechnol. 1997;15(6):553-7. [PMID: 11075358]. https://doi.org/10.1016/s0076-6879(00)28410-3.
49. Chen I, Dorr BM, Liu DR. A general strategy for the evolution of bond-forming enzymes using yeast display. Proc Natl Acad Sci U S A. 2011;108(28):11399-404. [PMID: 21697512] [PMCID: PMC3136257]. https://doi.org/10.1073/pnas.1101046108.
50. Song L, Zhang Y, Yun NE, Poussard AL, Smith JN, Smith JK, et al. Superior efficacy of a recombinant flagellin:H5N1 HA globular head vaccine is determined by the placement of the globular head within flagellin. Vaccine. 2009;27(42):5875-84. [PMID: 19654064] [PMCID: PMC3571653]. https://doi.org/10.1016/j.vaccine.2009.07.060.
51. Cho KJ, Schepens B, Moonens K, Deng L, Fiers W, Remaut H, Saelens X. Crystal Structure of the Conserved Amino Terminus of the Extracellular Domain of Matrix Protein 2 of Influenza A Virus Gripped by an Antibody. J Virol. 2016;90(1):611-5. [PMID: 26468526] [PMCID: PMC4702545]. https://doi.org/10.1128/jvi.02105-15.
52. Lei H, Jin S, Karlsson E, Schultz-Cherry S, Ye K. Yeast Surface-Displayed H5N1 Avian Influenza Vaccines. J Immunol Res. 2016;2016:4131324. [PMID: 28078309] [PMCID: PMC5204078] https://doi.org/10.1155/2016/4131324.
53. Kieke MC, Cho BK, Boder ET, Kranz DM, Wittrup KD. Isolation of anti-T cell receptor scFv mutants by yeast surface display. Protein Eng. 1997;10(11):1303-10. [PMID: 9514119] https://doi.org/10.1093/protein/10.11.1303.
54. Cho KJ, Schepens B, Seok JH, Kim S, Roose K, Lee JH, et al. Structure of the extracellular domain of matrix protein 2 of influenza A virus in complex with a protective monoclonal antibody. J Virol. 2015;89(7):3700-11. [PMID: 25609808] [PMCID: PMC4403401]. https://doi.org/10.1128/jvi.02576-14.
55. Zhang Y. I-TASSER server for protein 3D structure prediction. BMC Bioinformatics. 2008;9:40. [PMID: 18215316] [PMCID: PMC2245901]. https://doi.org/10.1186/1471-2105-9-40.
56. Berendsen HJ, van der Spoel D, van Drunen R. GROMACS: a message-passing parallel molecular dynamics implementation. Computer Physics Communications. 1995;91(1-3):43-56. https://doi.org/10.1016/0010-4655(95)00042-e.
57. Lindahl E, Hess B, Van Der Spoel D. GROMACS 3.0: a package for molecular simulation and trajectory analysis. Molecular modeling annual. 2001;7(8):306-17. https://doi.org/10.1007/s008940100045.
58. Eswar N, Webb B, Marti-Renom MA, Madhusudhan MS, Eramian D, Shen MY, et al. Comparative protein structure modeling using Modeller. Curr Protoc Bioinformatics. 2006;Chapter 5:Unit-5.6. [PMID: 18428767] [PMCID: PMC4186674]. https://doi.org/10.1002/0471250953.bi0506s15
59. Berendsen H, Postma JPM, van Gunsteren W, Hermans J. Interaction Models for Water in Relation to Protein Hydration. 111981. p. 331-42. https://doi.org/10.1007/978-94-015-7658-1_2158.
60. Parrinello MRA, Rahman AJ. Polymorphic Transitions in Single Crystals: A New Molecular Dynamics Method1982. 7182-90 p.
61. Bussi G, Donadio D, Parrinello M. Canonical sampling through velocity rescaling. J Chem Phys. 2007;126(1):014101. [PMID: 17212484]. https://doi.org/10.1063/1.2408420.
62. Hess B, Bekker H, Berendsen H, G. E. M. Fraaije J. LINCS: A Linear Constraint Solver for molecular simulations1998. https://doi.org/10.1002/(sici)1096-987x(199709)18:12<1463::aid-jcc4>3.0.co;2-h.
63. Humphrey W, Dalke A, Schulten K. VMD: visual molecular dynamics. J Mol Graph. 1996;14(1):33-8, 27-8. [PMID: 8744570]. https://doi.org/10.1016/0263-7855(96)00018-5.
64. Kumari R, Kumar R, Lynn A. g_mmpbsa--a GROMACS tool for high-throughput MM-PBSA calculations. J Chem Inf Model. 2014;54(7):1951-62. [PMID: 24850022]. https://doi.org/10.1021/ci500020m.
65. Shuid AN, Kempster R, McGuffin LJ. ReFOLD: a server for the refinement of 3D protein models guided by accurate quality estimates. Nucleic Acids Res. 2017;45(W1):W422-w8. [PMID: 28402475] [PMCID: PMC5570150]. https://doi.org/10.1093/nar/gkx249.
66. Brenke R, Hall DR, Chuang GY, Comeau SR, Bohnuud T, Beglov D, et al. Application of asymmetric statistical potentials to antibody-protein docking. Bioinformatics. 2012;28(20):2608-14. [PMID: 23053206] [PMCID: PMC3467743]. https://doi.org/10.1093/bioinformatics/bts493.
67. DeLano WL. PyMOL: An Open-Source Molecular Graphics Tool2002. 82-92 p.
68. Nili H, Asasi K. Avian influenza (H9N2) outbreak in Iran. Avian Dis. 2003;47(3 Suppl):828-31. [PMID: 14575072]. https://doi.org/10.1637/0005-2086-47.s3.828.

69. Javadmanesh A, Mohammadi E, Mousavi Z, Azghandi M, Tanhaiean A. Antibacterial effects assessment on some livestock pathogens, thermal stability and proposing a probable reason for different levels of activity of thanatin. Scientific Reports. 2021;11(1):10890. [PMCID: PMC8149819]. https://doi.org/10.1038/s41598-021-90313-4.
70. Wang W-C, Sayedahmed EE, Sambhara S, Mittal SK. Progress towards the Development of a Universal Influenza Vaccine. Viruses. 2022;14(8):1684. [PMID: 36016306] [PMCID: PMC9415875]. https://doi.org/10.3390/v14081684
71. Salleh AB, Rahim A, Rahman R, Leow TC, Basri M. The role of Arg157Ser in improving the compactness and stability of ARM lipase. J Comput Sci Syst Biol. 2012;5(2):39-46. https://doi.org/10.4172/jcsb.1000088.