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Research Article

Generation of New M2e-HA2 Fusion Chimeric Peptide to Development of a Recombinant Fusion Protein Vaccine

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Abstract

Purpose: The purpose was to design a new construction containing influenza virus (H1N1) M2e gene and HA2 gene by bioinformatics approach, cloning the construct in to Escherichia coli and produce M2e-HA2 peptide.

Methods: The procedure was done by virus cultivation in SPF eggs, hemagglutination assay (HA), RNA isolation, RT-PCR, primers designed (DNAMAN 4 and Oligo7), virtual fusion construction translation (ExPASy), N-Glycosylated sites prediction (Ensemblegly-Iowa), complete open reading frame (ORF), stop codon studied (NCBI ORF Finder), rare codon determination (GenScript), Solvent accessibility of epitopes (Swiss-PdbViewer), antigenic sites prediction (Protean), fusion PCR of M2e-HA2 gene, sequence analysis, nested PCR, gel electrophoresis, double digestion of pET22b(+) plasmid and the fusion construct, ligation of them, transformation of the ligated vector (pET22b-M2e-HA2) to *E.coli* (BL21), mass culture the cloned bacterium ,induction the expression by isopropyl-beta-D-thiogalactopyranoside (IPTG), sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), purification the fusion peptide by Ni-NTA column, western blot to verify the purification.

Results: In this study we developed a new approach for fusion of Influenza virus M2e (96 nucleotides) and HA2 (663 nucleotides) genes based on fusion PCR strategy and produced a fused fragment with 793 nucleotides. The construct was successfully cloned and expressed.

Conclusion: This construct is a 261 amino acid chimeric fusion peptide with about 30 KD molecular weight. According on the latest information; this is the first case of expression and purification M2e-HA2 fusion chimeric peptide, which could be used for development of a recombinant M2e-HA2 fusion protein vaccine.

Introduction

Influenza virus is an enveloped single-stranded negative-sense RNA virus, belongs to the *Orthomyxoviridae* family. This virus causes an acute and highly contagious respiratory disease. Seasonal epidemics of influenza disease cause serious illnesses and morbidity worldwide every year. Seasonal influenza each year: affects 5 to 15 percent of the world population and causes 3 to 5 million serious infections. It is responsible for the death of up to 500 thousand patients worldwide.[1,2] Pandemic is the other face of this disease. Based on a prediction, possible pandemic of this disease would cause, up to one billion infections.[3] According to the constant threat of a flu pandemic there is an urgent and serious need for developing antiviral strategies against the diverse influenza A viruses. Effective vaccination is one of the major ways to deal with the seasonal flu and the pandemic of this disease.

Two major characterization of influenza virus; the error-prone polymerase and segmented genome cause antigenic drift and shift respectively. Antigenic drift induces variation in surface glycoproteins of virus, especially in hemagglutination (HA) and to a lower extent in neuraminidase (NA). Antigenic drift makes seasonal influenza vaccines inefficient. Antigenic shift causes genome segments dealing between the different subtypes of the virus and makes new genetic combinations. Antigenic shift could lead to terrible pandemics by emerging new viruses that can be transferable between different species.[4]

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The need for a vaccine protecting human against all subtypes of influenza viruses including the causes of future pandemics leads to a new universal influenza vaccine approach. To design such a vaccine, conserved areas of the virus proteins are targeted. The candidate proteins should be protected from the influence of genetic drift and yet can induce effective protection against influenza virus subtypes. External matrix protein 2 (M2e), hemagglutination 2 (HA2) and nucleoprotein (NP) are the most conserved proteins of the influenza virus A which have the previously told features.[5-7]

M2 with 97 residues is the smallest protein of influenza virus. This type 3 trans- membrane protein acts as a pH regulated proton channel.[8,9] This function is effective in the beginning of the cell infection and virus progeny formation. M2e the external domain of the M2, only contains 32 amino acid residues. The unique characteristics of this domain, is that it is highly conserved, e.g. human influenza virus M2e has only up to five different position with avian M2e and therefore M2e human immune serum has cross react with almost all varieties of avian M2e.[10] The eight N-terminal residues of M2e (residues 2 to 9, SLLTEVET) is conserved in all subtypes of influenza A viruses.[11]

HA with 556 residues is the most abundant protein on influenza virus envelope. Cellular proteases cleave its precursor HA0 to HA1 and HA2, the cleavage is involved in the binding of virus to the cells surface, before entry into the cells.[12] The HA2 subunit (221 amino acids) has a hair spin-like structure composed of two antiparallel alpha-helixes. HA2 is more conserved than HA1, The hydrophobic N-terminal of the HA2 namely fusion peptide, especially its first 11 residues, is conserved among all influenza virus subtypes.[11] It has been shown that antibodies against HA2 can protect mice against the challenge with viruses from different clades.[13]

A suggested trend in the design of Universal flu vaccines is constructing of fusion proteins based on a combination of different conserved epitopes of influenza virus proteins. This approach can preserve universality features and improve the safety and protection of the vaccine. Moreover, such an approach compared to vaccines targeting a single subunit antigen, reduces time and cost of manufacturing.[11]

Based on the foregoing, considering well conserved sequences of M2e and HA2 and deficiencies in immunity induction in the use of single epitope (M2e or HA2), appears that the chimeric antigens of these two, can features an effective universal influenza vaccine. In this present study we designed a new structural model containing HA2 gene partial coding sequences (cds) and M2e gene partial cds which are fused together by a small linker. The design was done on base of bioinformatics studies. The designed fusion construct then cloned and expressed in a strain of *E. coli*.

Materials and Methods

Materials

LB broth medium (Lennox), Ampicilin powder were purchased from Sigma-Aldrich Company. dNTPs, T4 DNA ligase, *Nco*I and *Hind*III restriction endonucleases, Taq and Pfu DNA polymerase, GeneRuler™ 100 bp Plus DNA Ladder and GeneRuler™ 1Kbp DNA Ladder were purchased from Fermentas Company. Proteinase K, SDS, RNase A and lysozyme were purchased from CinnaGen. TriPure® Isolation Reagent, High Pure PCR Product Purification Kit and alkaline phosphatase enzyme were purchased from Roche Company. QIAprep Spin Miniprep Kit was purchased from Qiagen Company. pET22b(+) vector was purchased from Novagen. All other reagents were of analytic purity.

Virus and cell strain

Influenza virus used in this study was A/Brisbane/59/2007-like (H1N1), provided from NIBSC-UK. *E. coli* strain BL21 was purchased from Pasteur institute of Iran. SPF fertilized chicken eggs were prepared from Razi vaccine and serum research institute.

Cultivation

The virus (EID_{50} 10^5) was injected into the amniotic cavity (100µl) and allantoic cavity (100µl) of 10 days' specific pathogen free (SPF) embryonated chicken eggs, placed in a humidified incubator in 37°C for 2 days. After overnight chilling at 4°C, the allantoic and amniotic fluid of the eggs harvested .To prove the virus replication and measure its titer, hemagglutination assay (Ha) test was done on them.[14]

Total RNA Isolation and RT-PCR

Because of involving a spliced mRNA in encoding M2e, total RNA extraction is better than the only viral RNA isolation. After harvesting the allanto-amniotic fluid of the injected eggs and doing HA test for influenza virus, total RNA was extracted from the allanto-amniotic fluid using a nucleic acid isolation and Purification kit, (RocheTriPure® Isolation Reagent). The process was adapted to RNA extraction by adding RNA carrier in the process according to the manufacturer's recommendation.

The amplification of RNA requires the conversion of the RNA substrate into DNA, this is achieved through the use of a reverse transcriptase such as Moloney murine leukemia virus reverse transcriptase (M-MuLV RT) enzymes. Because of need for M2e spliced mRNA, we used $Oligo(dT)_{12-18}$ Primer(Invitrogen™). The primer hybridizes to the poly(A) tail of mRNA. All reactions were assembled on ice. 7µg of total RNA, 1µL $Oligo(dT)_{12-18}$ primers (0.5 µg/µL) ,4µL Nuclease-free dH_2O, were mixed in a nuclease-free tube. To denature RNA secondary structure, the tube was incubated at 70°C for 10 minutes and Placed on ice immediately. After a brief centrifugation, 4µL 5X Reverse Transcription Buffer, 2µL 0.1M DTT, 1µL 10mM dNTPs were added to the tube and mixed. After incubating at room temperature (~25°C) for 10 minutes, it was incubated at 42°C for 2 minutes. Then 1µL

RNase H- Reverse Transcriptase (200 units/µL) was added and to extend the RT reaction, incubated at 42°C for 50 minutes. To inactivate the enzyme, the tube incubated at 70°C for 15 minutes. Reactions diluted 5-fold by adding 80µL of nuclease-free dH_2O and Stored at ≤ -20°C.

Bioinformatics Analysis and Primers Design

Genetic Sequences

Genetic reference sequences of M2e (Accession No. CY058490.1) and HA2 (Accession No. CY058487.1) from A/Brisbane/59/2007-like (H1N1),retrieved from GenBank Databases.[15]

Fusion Construction Translation

Translation of the M2e-HA2 fusion construction to the protein was done by Translate online tool of ExPASy server.[16]

N-Glycosylated sites

In N-Glycosylated sites, carbohydrate chains attach to nitrogen of asparagine. N-linked glycosylation is the most common type of glycosidic bond and is important for the folding of some eukaryotic proteins and occurs in eukaryotes but very rarely in bacteria. We designed final expression of construct in *E. coli.* Therefore, it necessary to determine the epitopes that have N-Glycosylated sites. The ensemblegly- Iowa server[17] was used for prediction of N-Glycosylated sites.

Open Reading Frame (ORF)

The fusion structure was studied for complete ORF and stop codon by online NCBI ORF Finder online service.

Rare codon determination

Evaluation of final construct for rare codon analysis is a very important step because of its role in gene expression. For this purpose, rare codon analysis tools deposited in GenScript′s server[18] was used. This tool reads the input protein coding DNA sequence (CDS) and calculates its expressing organism related properties, like CAI (Codon Adaptation Index) and GC content.

Solvent accessibility of epitopes

Each residue at the surface of a protein can potentially be touched by water, and the area of an atom on the surface that can be touched by water is called the accessible molecular surface, or solvent-exposed area. Solvent accessibility of the fusion construct studied by" Swiss-PdbViewer" software.

Antigenic sites prediction

As the final goal of the study was to produce an immunogenic fusion peptide, antigenic sites prediction was very important. It was done by the Protean software from Laser gene 12.1 (DNASTAR) package.

Primers Design

DNAMAN 4 and Oligo7 were used to design primers needed to isolate and fusion of M2e and HA2 gene segments and nested PCR primers.

PCR amplification of M2e and HA2 genes

Polymerase chain reactions (PCRs) were carried out in 50µl mixtures for M2e and HA2 separately, containing 5µl of 10x reaction buffer with magnesium sulfate (MgSO4), 4µl of mixed deoxyribonucleotide triphosphats (dNTPs) (2.5mM each), 1µl of each specific forward and reverse primers (10pmol each), 0.5µl of Pyrococcus furiosus (pfu) DNA polymerase(2.5u/µl) (Fermentas Company), 3µl cDNA template and 35.5µl of diethyl pyrocarbonate (DEPC) water. Thirty five cycles of PCR were performed (denaturation at 94°C for 3 min, annealing at 61°C for 1 min, and extension at 72°C for 2 min).

Sequence analysis of amplified and purified PCR products

The PCR products in the size of 118b and 711b were excised from agarose gel following electrophoresis, and purified by using the *High Pure PCR Product Purification Kit* (Roche) according to the manufacturer's recommendations. Nucleotide sequencing was carried out by *Macrogen Korea*.

M2e-HA2 chimeric fusion gene construction

Fusion PCR was carried out for *M2e-HA2* chimeric fusion gene construction. Purified products of HA2 and M2e genes PCR amplifications were used in a two steps PCR reaction. The 1st step reaction was designed as a 3 cycle reaction and started in a 0.2 ml thin walled micro tube using all needed components except of primers. After 3 cycles M2e forward and HA2 reveres primers were added to the same micro-tube and 2nd step of PCR reaction was continued for 20 more cycles(denaturation at 94°C for 3 min, annealing at 61°C for 1 min, and extension at 72°C for 2 min).The blunt end PCR product in the size of 793b (fusion gene) was excised from agarose gel following electrophoresis, and purified by using the *High Pure PCR Product Purification Kit* (Roche) according to the manufacturer's recommendations.

Verification of M2e-HA2 fusion gene construction by internal primers

Nested PCR was carried out for M2e-HA2 fusion gene construction using the sequence of this fusion gene. These internal primers are as followed, the product is from nucleotide number 28 to nucleotide number 247 that include parts of both the M2e and HA2, and product size was 200 bp. Forward nested primer(from 28 to 48), Reverse nested primer (from 227 to 247).

Cloning of M2e-HA2 chimeric fusion gene

pET-22b+(Novagen) was selected as a cloning and expression vector with ampicillin resistant gene and His-tag coding sequence, The plasmid and purified M2e-HA2 fusion gene were double digested separately, by *Nco*I and *Hind*III (Fermentas). The reaction contained: 3µg of vector or PCR product, 3µl of 10x universal restriction enzyme buffer, 3µl for each restriction enzyme, 21µl nuclease free water, incubated at 37°c for overnight. To prevent self-ligation, 0.3µl of one unit/µl

alkaline phosphatase(Roche)was added and incubated at 37°C for 30 minutes. For ligation reaction we used;25ng double digested *pET-22bplus*, 75ng double digested M2e-HA2 fusion gene, 10X Ligase Buffer 1µL/10µL, 1µL T4 DNA Ligase (Fermentas) and H_2O to a total of 10µL, Incubated at room temperature for 2hr.

Transformation

We used heat shock method for transforming our ligated vector to the calcium competent bacterial cells. *E. coli* strain BL21 that is ideal for high-efficiency cloning and expression was used to produce competent cells and its efficiency tested. Ligation mixture (10µl) directly used for transformation of 100µl of calcium competent bacterial cells. The transformed cells were cultured on a plate containing LB agar medium and 100µg/ml ampicillin and incubated at 37°C for one night. The complete technique was done as described previously.[19]

Verification of fusion construction

Because of ampicillin in LB agar plates the grown colonies likely containing recombinant *pET-22b+* plasmids. After one night, colony PCR was carried out for the colonies grown on the LB plates. The same colony was cultured on a new plate containing LB agar medium plus ampicillin. M2e Forward primer and HA2 Reverse primer were used for the reaction. The reaction was done as mentioned above except that a small amount of the colony added instead of the template. For negative control pET-22b+non recombinant plasmid vector transformed to *E. coli* strain BL21 competent and a colony of this bacteria also was subjected to the same PCR procedure. One *E. coli* strain BL21 competent but not transformed colony, also cultured on the same plate to control the ampicillin efficacy. Plasmid extraction carried out by QIAprep Spin Miniprep Kit (Qiagen). Plasmid digestion was done using NcoI and HindIII restriction endonuclease according to Fermentas protocol. Two set of PCR was carried out for M2e-HA2 fusion gene construction using purified plasmid. Internal M2e-HA2 fusion gene reverse and forward primers were used for nested PCR. All of the above recombinant plasmids and PCR products analyzed after separation by agarose gel electrophoresis.

M2e-HA2 recombinant fusion peptide expression

100µl of transformed cells stock was cultures in10ml of 2xYT liquid medium containing 1% glucose and 100µg/ml Ampicillin for overnight in 30°C shaking incubator.2×300µl of the cultures were cultured in 2×5ml of 2xYT liquid medium containing 1% glucose and 100µg/ml Ampicillin for 2 hours in 30°C shaking incubator.

Each 5ml cultured medium were added to 500ml fresh 2xYT liquid medium containing 1% glucose and 100µg/ml Ampicillin and incubated in 30°C shaking incubator, when the optical density (OD) of the culture in 600nm were 0.8 they put in 4°C for 20 minutes. 10ml of the culture were taken as non-induced sample. 1mM of IPTG were used to induce expression and cultures put in 30°C shaking incubator for 6 hours.

M2e-HA2 recombinant fusion peptide purification

Sedimentation was done by centrifuging the cultures (in 250ml falcons) for 5 min at 600 rpm, washing procedure repeated 5 times with adding PBS. The pET-22b(+) vector carries an C-terminal His•Tag® that expressed as a 6× Histidine tail in the C-terminal of M2e-HA2 fusion peptide. These Histidines show affinity to Ni ions on the NTA matrix in Ni-NTA column. The purification procedure on NI-NTA column (Qiagen) was done according to the manufacturer's manual.

Verification the expression and purification

M2e-HA2 fusion peptide is a ≈30KD peptide, its expression was verified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as described previously.[20] Western blot was done on the purified samples against His-tag by using Anti-6X His tag® antibody (Abcam) as described previously.[21]

Results

Design of a fusion construct containing M2e and HA2 gene segments of influenza virus A/Brisbane/59/2007-like (H1N1) were studied by bioinformatics approach. Amplification of both of the M2e and HA2 gene segments were done by designed primers and the fusion construct composed of M2e, a small linker and HA2 include restriction sites and expression inducer sequences was made by fusion PCR strategy (Figure 1). Verification of PCR products and final fusion construct were done by agarose gel electrophoresis and sequencing.

Bioinformatics Analysis Results

M2e peptide is the production of a spliced mRNA include nucleotide 1 to 26 and 715 to 757 of 7th segments of influenza virus genome. HA2 is a 666 nucleotides fragment of 4th segments of influenza virus genome, from 1030 to 1695.

The ensembleGly server result for our translated construction showed only 3 potentially N-Glycosylated sites.

Possibility of high protein expression level is correlated to the value of CAI, a CAI of >0.8 is rated as good for expression in the desired expression organism. Our construction CAI was about 0.72. The ideal percentage range of GC content is between 30% to 70%. Average of our construct GC content was about 40.7%.

The fusion structure was studied by online NCBI ORF Finder software, there was a complete ORF and there was not any stop codon.

The antigenicity of construct analyzed by Protean software from Laser gene package The plot showed that major parts of the fusion protein construct have hydrophilic property thus harbor antigenic potency (Figure 2).

M2e gene forward primer, contained of NcoI site and an earring. M2e reverse primer contained reverse complementary of the linker sequence (Table 1). For HA2 forward primer, we used 18 bases of the reverse complementary of the template end and added the linker sequence. We used reverse complementary of the last 17 bases of the template for the reverse primer and added a restriction site for HindIII and an earring to the end of it. Internal primers for nested PCR were designed on bases of the fusion construction sequences, the forward primer from nucleotide 28 to 48 and the reverse primer from nucleotide227 to 247 (Table 1).

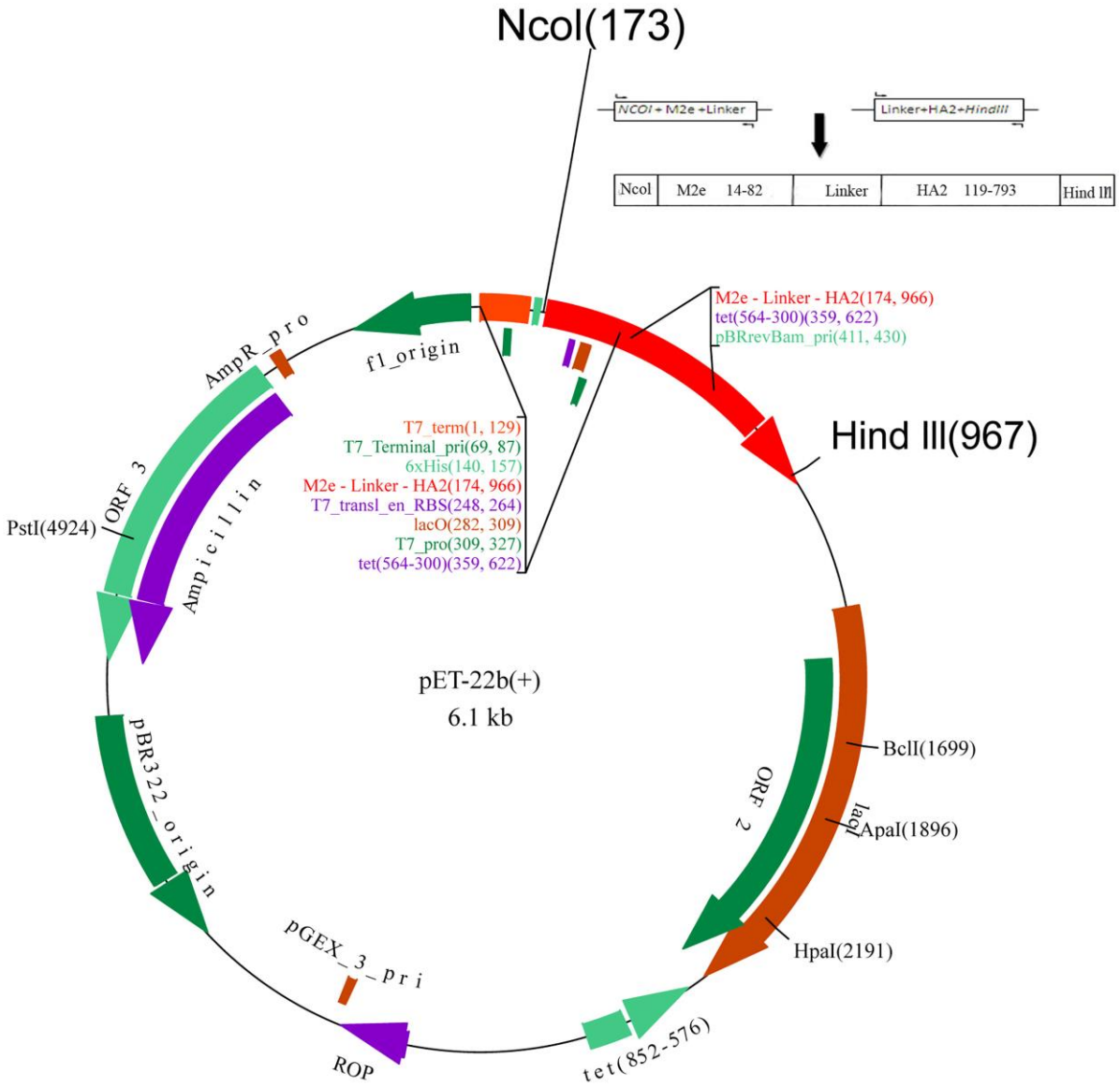

Figure 1. M2e-HA2 fusion construction and pET22b+ details: M2e segment consist of NcoI recognition site and the linker, HA2 segment consist of the linker and the HindIII recognition site. The fusion construct inserted between the NcoI and HindIII recognition sites of the pET-22b.

Sequencing analysis of amplified and purified PCR products

Sequencing data of influenza virus A/Brisbane/59/2007-like (H1N1) M2e and HA2 genes showed118bp and 711bp length, respectively. Sequencing results of PCR amplified M2e and HA2 genes showed complete identities between these two genes.

M2e-HA2 chimeric fusion gene construction

At the next step M2e forward and HA2 reveres primers were used to produce a chimeric gene containing amplified partial cds of M2e and partial cds of HA2, which were linked together by the AEAAAKEAAAKA sequence as a small linker. The fusion construction length (include distal and proximal earrings and restriction sites, M2e gene, linker and HA2 gene) was 793bp. The fusion PCR method was done, based on fusing the two complementary strands of linker on the PCR products of first PCR (M2e and HA2) and using of Pfu DNA polymerase, which has a proofreading activity (Figure 3).

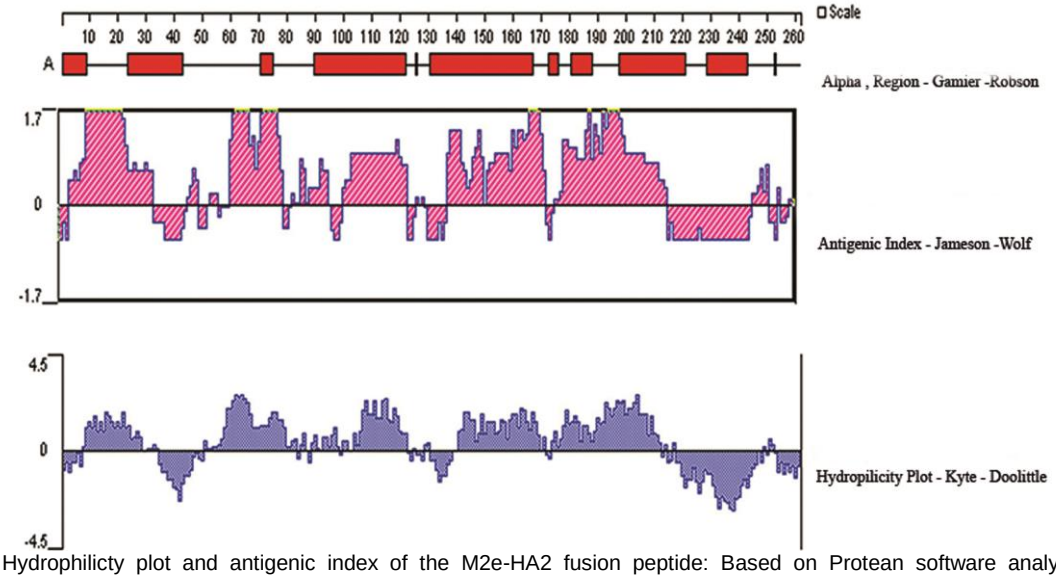

Figure 2. Hydrophilicty plot and antigenic index of the M2e-HA2 fusion peptide: Based on Protean software analyzing potential antigenicity of the construct is shown. The majority of its length had the potential for antigenicity.

Table 1. Primers sequences: M2e Forward primer consisting NcoI recognition site and an earring, M2e Reverse primer consisting the linker sequence, HA2 Forward primer consisting the linker sequence, HA2 Reverse primer consisting HindIII recognition site and an earring, Forward nested primer and Reverse nested primer for the fusion construction.

Primer	Restriction enzyme sites, linker and nested sequences
M2e Forward primer	*Nco*I 5'CATG*C*/*CATGG*GTCTTCTAACCGAG3'
M2e Reverse primer	Linker 5'TGCTTTCGCCGCGGCTTCCTTCGCCGCAGCCTCTGCATCACTTGAATCGTTGCATC3'
HA2 Forward primer	Linker 5'GCAGAGGCTGCGGCGAAGGAAGCCGCGGCGAAAGCAGGTTTGTTTGGAGCCATTG3'
HA2 Reverse primer	*Hind* III *5*'CCCA/AGCTTGATGCATATTCTACACTGTA3'
Forward nested primer	*5'GGTCGAAACGCCTATCAGAA3'*
Reverse nested primer	*5' CTGCAGCATAGCCAGATCCT 3'*

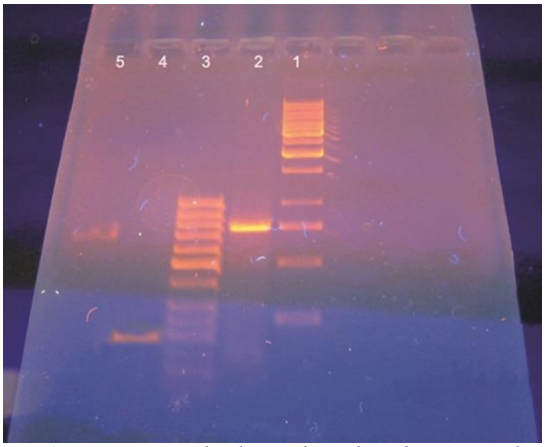

Figure 3. Agarose gel electrophoresis of M2e and HA2 segments and fusion PCR products: M2e and HA2 genes showed respectively about 100 and 700bp. The fusion construction length (include distal and proximal earrings and restriction sites, M2e gene, linker and HA2 gene) was about 800 bp. 1= 1kb ladder, 2= fusion PCR product, 3= 50bp ladder, 4= M2e, 5= HA2

Verification of M2e-HA2 fusion gene

Verification of the construction was done by internal primers. The length of amplified fragment between inner forward reveres primers which used for nested PCR, including parts of both the M2e and HA2, and product size was 200 bp (Figure 4).

Cconstruction of M2eHA2-pET22 vector and its Transformation

During ligation M2e-HA2 fusion gene inserted into *pET-22b+*(5446bp linear) vector at the blunt ends and produced a 6239bp circular recombinant plasmid, Figure 4 shows recombinant *pET-22b+*vector diagram. Overnight culture of *E. coli* strain TOP10 which had been transformed directly with this ligation product, showed colonies and good condition of growth on LB agar containing ampicillin. While *E.coli* strain *BL21* non transformed competent colony did not grown on the LB agar with ampicillin. Extracted undigested recombinant cloning vector showed two sharp bands above 5kb on electrophoresis (Figure 5).

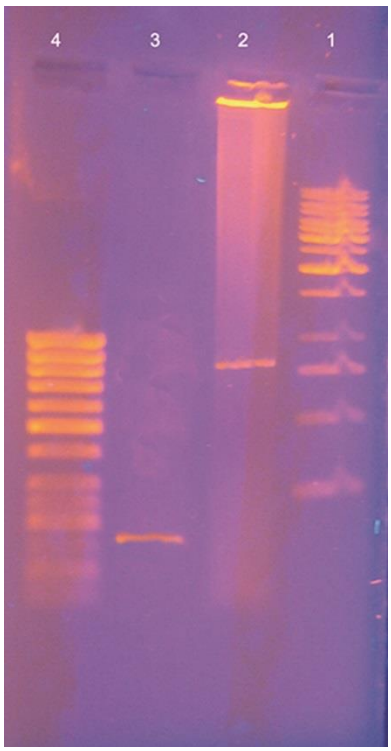

Figure 4. Gel electrophoresis of the fusion construction nested PCR products. PCR product of recombinant cloning vector using internal forward and reveres primers showed one sharp 200bp band but the fusion construction primers amplified~800 bp PCR product. 1= 1kb ladder, 2= fusion construction, 3= nested product, 4= 50 bp ladder

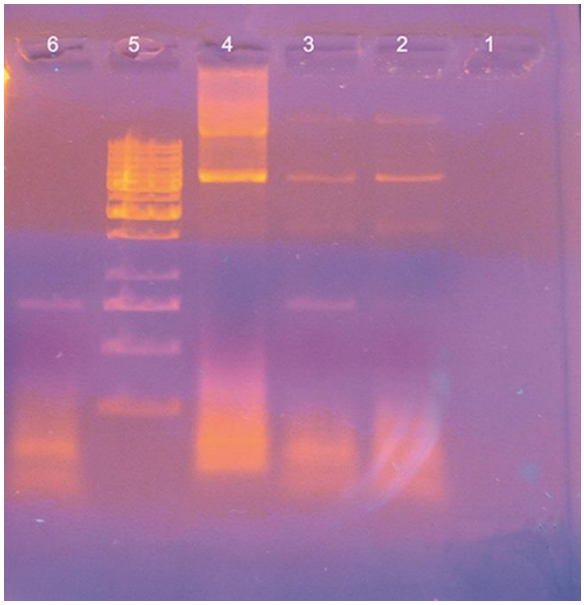

Figure 5. Digested and indigested recombinant plasmid: This agarose gel electrophoresis was done to verify the fusion construct insertion in pET-22b. Negative control PCR product showed no bond. Verification of fusion construction. Digested recombinant cloning vector by NcoI and HindIII restriction endonucleases showed one sharp~ 800 bp and one sharp 5.5 kb bands. 1= negative control, 2= undigested recombinant plasmid, 3= digested recombinant plasmid, 4= undigested plasmid, 5= 1kb ladder, 6= fusion construct.

Verification of fusion construction

PCR product gel electrophoresis of recombinant plasmid colony PCR showed ~800 bp DNA fragment. Negative control PCR product showed no bond. Culture result of the same colony on a LB agar plate containing ampicillin, showed good and suitable growth of bacteria. When this recombinant cloning vector was digested by *Nco*I and *HindIII* restriction endonucleases showed one sharp~800 bp and one sharp 5.5 kb (Figure 5). PCR product of recombinant cloning vector using internal forward and reveres primers showed one sharp 200bp band but our primer amplified~800 bp PCR product (Figure 5).

Expression and purification of recombinant proteins

To verify protein expression, SDS PAGE analysis of the purified protein revealed a single band with a molecular weight of about 30KD (Figure 6). Western blot using anti-His tag monoclonal antibody were done on the transformed cells lysate and showed a ≈30KD band and confirmed the expression of the pET22b(+)/M2e-HA2 in the system(Figure 7). After purification the fusion peptide by Ni-NTA column (Qiagen) the peptide concentration measurement by Bradford assay, finish product contained a concentration of 25µg per ml of HA2-M2e.

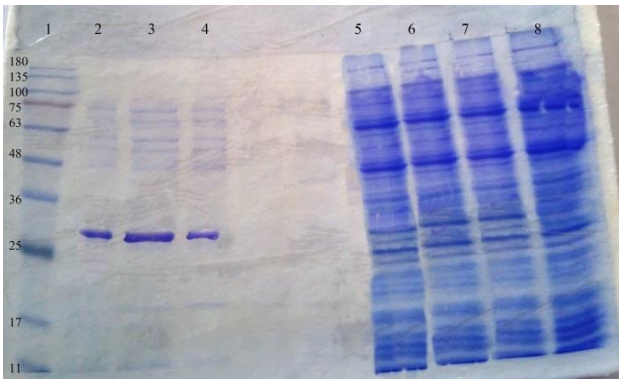

Figure 6. SDS-PAGE analysis of induced and purified samples: an about 30KD band could be seen in purified samples (2,3&4). In induced but not purified samples the band could be seen among other bands (5,6&7). In non-induced sample there is not distinguished ≈30KD band. 1= protein ladder. 2,3 and 4= purified samples. 5,6 and7= induced sampled. 8= non induced sample.

Discussion

Influenza virus antigens bear major and minor changes namely antigenic shift and drift respectively. These changes lead to inefficacy of the vaccines through time and against different influenza virus subtypes. There is a need for universal vaccines to protect human against all subtypes of influenza viruses include future pandemics which its protection would not impressed by antigenic shift and drift. Conserved viral proteins such as M2e, HA2 and NP are the targets for this approach.[5-7]

M2e peptide contains 32 amino acid residues. It is highly conserved, e.g. M2e human immune serum has cross react with almost all varieties of avian M2e.[10] There are some reports about the M2e immunogenicity against

influenza virus.[22,23] Schotsaert *et al* (2009) have demonstrated the safety and immunogenicity of M2e-based vaccine in a phase I clinical studies.[24] HA2 subunit with 221 amino acids is a conserved peptide. The 11 first residues of HA2 N-terminal is conserved among all influenza virus subtypes.[11] Bommakanti G. et al (2010) expressed HA2 in *E. coli*.[13] There are some reports about the HA2 immunogenicity against influenza virus.[25,26] The HA2 molecule is larger than M2e and also is more immunogenic. Maybe fusions of these two peptides can increase synergetic effects on the universality and immunogenicity of both of them. Generally cloning and expression of a proper designed fusion construction is easier and cheaper than two single constructs. The production of Influenza virus antigens in cheap and suitable host, such as *E. coli* could be the answer to the egg based vaccine production problems such as, the vulnerability of chicken flocks in an influenza pandemic and the long period required for vaccine seeds to egg adaptation.[7] In this study a new construction containing influenza virus A/Brisbane/59/2007-like (H1N1) M2e gene partial cds and HA2 gene partial cds was designed to express in *E. coli*.

Maybe the main problems in production of antigens, in a host such as *E. coli* are proper expression of the protein with right folding in large amounts and easy purification of it. Glycosidic bonds have important role in proper folding of eukaryotic proteins. Glycosylation occurs in eukaryotes and expression of a peptide with much glycosylated sites in a prokaryote host can result in improper folding. Selected areas for the fusion construction had least glycosylated sites (only 3 sites, 20th, 200th, 259th amino acids). According to the antigenicity index of the fusion peptide amino acids (Figure 2) which these sites are not very important in the peptide antigenicity. To avoid interfere between M2e and HA2 antigens we used a linker with 36 bp (nucleotides 83-118 of the fusion construction), 12 amino acid (AEAAAKEAAAKA) that is optimized for *E. coli*. The linker contained two repeating segments of EAAAK between two As. A, Alanine is a nonpolar amino acid, E, Glutamine is a polar amino acid with positive charge and K, Lysine is a polar amino acid with negative charge. Salt bridges between E and K stabilized the helical linker by~80% helicity. This hydrophobic linker can provide proper flexibility and separation between M2e and HA2 antigens.[27,28]

In this study we developed a new approach for fusion of Influenza virus M2e and HA2 genes based on fusion PCR strategy. In conventional methods the steps are much and more complex.[28] Although making the fusion construct of *Clostridium perfringens* type D, Epsilon and Beta toxin genes based on fusion PCR strategy also is reported,[29] but according on the latest information, this is the first design by bioinformatics approach, cloning of M2e-HA2 fusion gene by PCR fusion strategy and also this is the first time that it is expressed in *E. coli* BL21 strain, which could be used for development of a recombinant M2e-HA2 fusion protein vaccine.

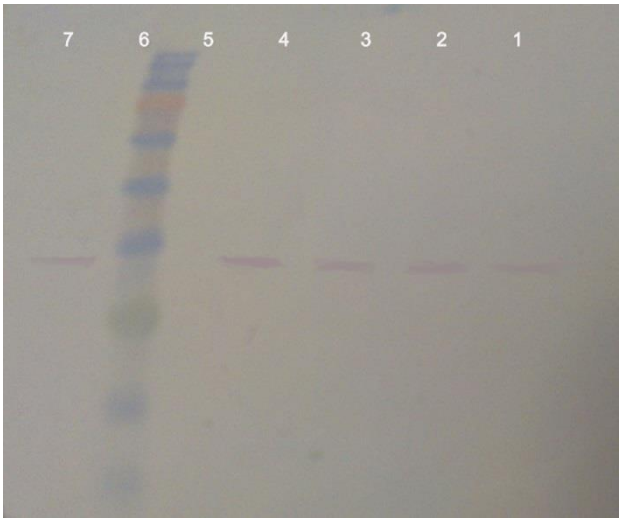

Figure 7. Western blot using anti-His tag monoclonal antibody: there is blots on about 30KD bands places in induced (1,2,3,4) and purified samples (7) but not in non-induced sample (5). 1,2,3 and 4=induced samples. 5= non induced sample, 6= pre-stained protein ladder. 7= purified sample.

Conclusion

To achieve a proper vaccine candidate, expression and clinical studies on this fusion construct and its product should be done. The areas for further research include immunization studies on animal models such as mice and chicken, homologous and heterologous influenza viruses challenge studies, use of the fusion peptide with different adjuvants and use the construct as a DNA vaccine.

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Ethical Issues

Not applicable.

Conflict of Interest

The authors report no conflicts of interest.

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