

Fusion and sequence analysis of the influenza A (H9N2) virus M2e and C-terminal fragment of *Mycobacterium tuberculosis* HSP70 (H37Rv)

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ABSTRACT

The present study was aimed to construct a fusion plasmid harboring the extracellular domain of the influenza A M2-protein (M2e), which was fused to the N-terminus of the truncated HSP70 (HSP70₃₅₉₋₆₁₀) molecule as a new approach for future vaccine research against influenza A. The amplified fragments, *M2e* and *HSP70*₃₅₉₋₆₁₀ genes, were gel-purified. The products were then single digested with *Bam*HI restriction enzyme separately. The digested products were again gel-purified and ligated by *T4* DNA ligase to form *M2e*-*HSP*₃₅₉₋₆₁₀ gene. The PCR product containing both *M2e* and *HSP*₃₅₉₋₆₁₀ genes as a single open reading frame (ORF) was gel-purified and double digested with *Eco*RI and *Xba*I restriction enzymes and then ligated into the *Eco*RI / *Xba*I double digested pPICZαA expression vector to form recombinant expression vector. Finally, the fused gene was sequenced, and then confirmed according to the related deposited gene in Genbank. The extracellular domain of the M2 protein, M2e, which consists of N-terminal 24 residues, showed to be remarkably conserved, and the N-terminal epitope SLLTEVET (residues 2-9) was conserved among all subtypes of influenza A viruses. Because of M2e limited potency; hence, low immunogenicity, it seems by linking this M2e-peptide to an appropriate carrier such as *mycobacterium tuberculosis* C-terminal 28-kDa domain of HSP70 (hsp70₃₅₉₋₆₁₀) we can render it very immunogenic, but further study needs to express it in both prokaryotic and eukaryotic systems and then evaluate this fusion protein in animal model.

Keywords: avian influenza, fusion, vaccine, HSP70, M2e

INTRODUCTION

The main option for reducing the impact of influenza is vaccination. In Iran, only inactivated

influenza virus vaccines are approved at this time. To be effective, current vaccines must contain an H1N1, an H3N2, and a B virus component. Over the past several years, at least one of the components in the formulation had to be changed due to antigenic drift of the strain circulating in the human population. The possibility of developing a

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universal influenza vaccine has attracted the attention of many researchers, because the continuing antigenic change of influenza viruses necessitates reformulating the vaccine on a nearly annual basis. The conserved extra-domain of influenza M2 protein (M2e) is considered as a promising candidate target for a broad-spectrum, recombinant influenza A vaccine (Neiryneck *et al* 1999). M2e is a 24 amino acid peptide. Possibly because of its small size and its location close to the membrane amidst numerous giant glycoproteins and glycolipids, it is hardly seen by the cells of the immune system and thus less likely to induce a protective response (Ebrahimi *et al* 2008; Ebrahimi *et al* 2009). Hence, optimal approaches to enhance immunogenicity of M2e protein immunization remain to be established. In recent years, various heat shock proteins (HSPs) and molecular chaperones have been proposed as adjuvant for cross priming with antigenic peptides (Qazia *et al* 2005; Chen *et al* 2000). HSP70 is a member of a family of molecular chaperones and highly conserved in evolution (Lindquist *et al.*, 1988). The antigenic nature of mycobacterial hsp70 allows it to be used as an adjuvant-free carrier molecule under certain circumstances. Wang *et al.* (2002) recorded two major fragments of hsp70: an N-terminal 44-kDa ATPase domain (hsp70 1–358) and a C-terminal 28-kDa domain (hsp70 359–610) that contains the 18-kDa peptide binding region (amino acid 359–540) (Wang *et al.*, 2002). Li *et al.* (2006) showed that only the HSP70₃₅₉₋₆₁₀- fused hepatitis B virus DNA vaccination resulted in a significant increase in hepatitis B surface antigen (HBsAg) - specific humoral response, while the HSP70₁₋₃₆₀- or the complete HSP70 molecule-fused vaccine did not. So, It seems by linking this M2e-peptide to an appropriate carrier such as *mycobacterium tuberculosis* C-terminal 28-kDa domain of hsp70 (hsp70₃₅₉₋₆₁₀) we can render it very immunogenic. Therefore, the present study was sought to construct a fusion plasmid harboring the extracellular domain

of the influenza A M2-protein (M2e), which was fused to the N-terminus of the truncated HSP70 (HSP70₃₅₉₋₆₁₀) molecule as a new approach for future vaccine research against influenza A.

MATERIALS AND METHODS

PCR amplification and DNA cloning. The *M2e* gene (72 bp) was PCR amplified from the previously described pAED4-M2 plasmid template [Ebrahimi *et al* 2009], which carries *M2* gene from avian influenza virus A/chicken/iran/101/98 (H9N2), using the upstream (5'-CCGGAATTCATGAGTCTTCTAACCGAG-3') and downstream (5'-CGCGGATCCATCACTTGAATCGCTGCA-3') primers that harbored the *EcoRI* and *BamHI* restriction sites (underlined sequences), respectively. PCR was performed in a 50 µl reaction mixture containing 10X buffer with 2mM MgSO₄, mixed dNTPs (2.5 mM each), specific primers (10 pmol each), 1.2 U of *pfu* DNA polymerase (Mannheim, Roche, Germany) and 100 ng of pAED4-M2 plasmid as template. Amplification program was set as 95 °C for 3 min, which was followed by 5 cycles of 95 °C for 1 min, 54 °C for 1 min, 72 °C for 1 min, and then 30 cycles of 95 °C for 1 min, 56 °C for 1 min, 72 °C for 1 min, and a final extension at 72 °C for 5 min. The resulting PCR products were then analyzed by 1.5% (w/v) agarose gel electrophoresis. The C-terminal domain of HSP70 (HSP70₃₅₉₋₆₁₀) was isolated from the genome of *Mycobacterium tuberculosis* (H37Rv) by PCR and using the specific upstream (5'-CGCGGATCCGAGGTGAAAGACGTTCT-3') and downstream (5'-GCTCTAGACTTGGCCTCCCGGCCGTC-3') primers, harboring *BamHI* and *XbaI* sites (underlined sequences), respectively. The PCR parameters were similar to the *M2e* program, except the annealing temperature that was set at 63 °C for 1 min.

Fusion of M2e gene to HSP70₃₅₉₋₆₁₀ as a single ORF. The amplified fragments, *M2e* and *HSP70*₃₅₉₋₆₁₀ genes, were gel-purified using high pure PCR product purification kit (Roche, German), according to manufacturer's protocol. The products were then single digested with *Bam*HI restriction enzyme separately. The digested products were again gel-purified and ligated by *T4* DNA ligase (Roche, Germany) to form *M2e*- *HSP*₃₅₉₋₆₁₀ gene. To minimize interference between adjacent proteins, each protein was separated from its neighboring one by a glycine and a serine codon (Figure 1). The PCR was performed on ligation mixture using *M2e* upstream and *HSP*₃₅₉₋₆₁₀ downstream primers. The PCR parameters were as above mentioned, but the melting temperature was 59 °C for 1 min.



Figure 1 Schematic representation of the fused *M2e* and *HSP70*₃₅₉₋₆₁₀ genes as a one open reading frame (~900-bp).

Construction of recombinant plasmid pPICMH.

The PCR product containing both *M2e* and *HSP*₃₅₉₋₆₁₀ genes as a single open reading from (ORF) was gel-purified and double digested with *Eco*RI and *Xba*I restriction enzymes (Fermentas, Germany) and then ligated into the *Eco*RI / *Xba*I double digested pPICZαA expression vector (Invitrogen, USA) to form recombinant expression vector (Figure 2). *E. coli* strain DH5α was transformed with the ligated vector and transformants were selected on low salt LB-agar plates containing 25 μg/ml Zeocin (Invitrogen, USA). Single colonies were selected and the sequence of the isolated plasmids was analyzed by the MWG Biotech Co. (Germany) to verify the presence of the correct and expected insert. The procedures for small scale preparation of plasmid, digestion with restriction enzymes, ligation

and transformation all followed according to manufacturer's protocols.

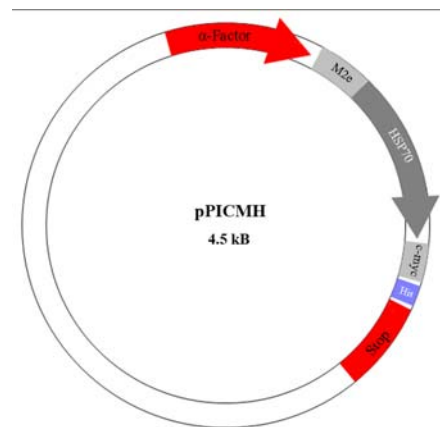


Figure 2. Schematic representation of the recombinant expression vector; pPICMH plasmid, that encoded *M2e*-*HSP70*₃₅₉₋₆₁₀-*Cmyc*-*His* fusion as a secretory protein based on the presence of α-factor signal sequence. P_{AOX1} denotes to the alcohol oxidase promoter and *Eco*RI, *Bam*HI and *Xba*I determine the endonuclease restriction sites.

RESULTS

PCR was able to amplify the desirable fragment (~100 bp; containing restriction site linker) of the influenza A virus *M2e* and *Mycobacterium tuberculosis* (H37Rv) *HSP70*₃₅₉₋₆₁₀ (~820 bp: containing restriction site linker) genes, then the *M2e* gene was successfully ligated into the N-terminal of *HSP70*₃₅₉₋₆₁₀ to form an *M2e*- *HSP70*₃₅₉₋₆₁₀ fused gene as a one open reading frame (ORF) at the size of about 900 bp (Figure 3). Then the fused gene was ligated into the multiple cloning site region downstream of the *P. pastoris* Aox1 promoter and the α-factor signal sequence of pPICZαA vector. The resulting plasmid was transformed into the *E. coli* strain DH5α; the positive clones were screened with colony PCR by the *M2e* upstream and the *HSP*₃₅₉₋₆₁₀ downstream primers. The PCR products were confirmed to contain ~900 bp as expected by agarose gel electrophoresis. Correct orientation was identified with the aid of the restriction analysis (RE) using

EcoRI and *XbaI* restriction enzymes. RE analysis of the clones gave the expected size of the two bands (~900 bp and 3.6 kb respectively) (Figure 4).

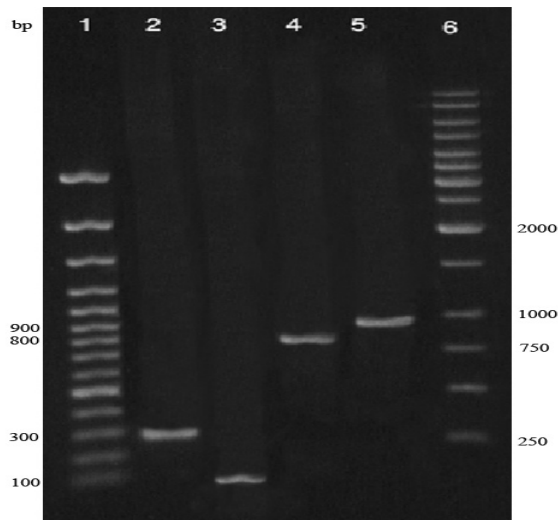


Figure 3 Analysis of the PCR products on 1.5 % (w/v) agarose gel. Lane 1, contains 100 bp ladder (Fermentas, Germany); lane 2, contains ~300 bp *M2* gene; lane 3, contains ~100 bp *M2* extracellular domain (*M2e*); lane 4, contains ~820 bp *HSP70*₃₅₉₋₆₁₀; lane 5, contains ~900 bp *M2e-HSP70*₃₅₉₋₆₁₀ (fusion); lane 6, 1kb ladder (Fermentas, Germany).

Finally, the fused gene was sequenced, the *M2e* gene sequencing showed 100% homology with the *M2* gene (1-72 bp) of the influenza A virus (H9N2) deposited in Genbank with accession number of **EU477375**. The *HSP*₃₅₉₋₆₁₀ gene sequencing also showed 100% homology with the *Mycobacterium tuberculosis* H37Rv *hsp70* gene (1075-1888 bp) deposited in Genbank with accession number of **BX842573** (Fig. 5). The amino acid sequence of the fusion *M2e* and *HSP*₃₅₉₋₆₁₀ gene as a single ORF has a predicted molecular weight of 31.13 kDa.

DISCUSSION

These days, the threat of highly virulent avian influenza, such as H5N1 and H1N1 viruses, brings out an urgent need to develop a universal influenza vaccine, which could provide cross-protection against different influenza virus strains (Gerhard *et al* 2006). The conserved extra-domain of influenza

M2 protein (*M2e*) is considered as a promising candidate target for a broad-spectrum, recombinant influenza A vaccine, as it is almost completely conserve across all influenza A virus specially H5N1 and H9N2 (Ebrahimi *et al* 2008) and several *M2*-based vaccines have been proved to provide successful protection against homologous and heterologous influenza virus challenge, including H5N1 subtype (Slepushkin *et al* 1995, Neiryneck *et al* 1999, Tompkins *et al* 2007, Ernst *et al* 2006).

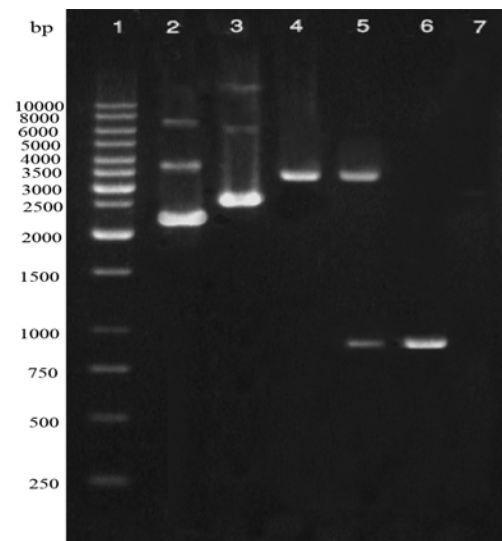


Figure 4. PCR and restriction enzyme analysis of the constructed pPICMH plasmid on 1.2 % (w/v) agarose gel. Lane 1: size marker 1 Kb (Fermentas, Lithuania); Lane 2: pPICZ α A plasmid without insert; Lane 3: pPICZ α A with insert (pPICZ α A-M2e-HSP70₃₅₉₋₆₁₀, pPICMH); Lane 4: pPICZ α A plasmid linearized by *EcoRI* enzyme; Lane 5: pPICMH double digested by *EcoRI* and *XbaI* enzymes, showing the ~900 bp insert; Lane 6: PCR amplification of insert on pPICMH template using specific primers; Lane 7: negative control.

In the present study, for the first time avian influenza virus *M2e* gene with C-terminal domain of *HSP70* of *mycobacterium tuberculosis* was successfully fused and then cloned as a single open reading frame (ORF) in pPICZ α A to be used as constructed secretary expression vectors in *Pichia* strains. The extracellular domain of the *M2* protein, *M2e*, which consists of N-terminal 24 residues, is remarkably conserved, and the N-terminal epitope SLLTEVET (residues 2-9) is conserved among all

subtypes of influenza A viruses. A prerequisite to the use of influenza M2e protein as a bivalent influenza vaccine candidate was to find out the differences between human and non-human (avian and swine) influenza virus M2e proteins.

As a result, the host specific sequences were identified based on influenza virus M2e protein: PIRNEWGCRCN, PTRNGWECKCS and PIRNGWECRCN (aa 10-20; human, avian and swine specific sequences, respectively).

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1      ATGAGTCTTCTAACCGAGGTCGAAACGCTTACCAGAAACGGATGGGGTTGCAGATGCAGC
1      M S L L T E V E T L T R N G W G C R C S

61     GATTCAAGTGATGGATCCGAGGTGAAAGACGTTCTGCTGCTTGATGTTACCCCGCTGAGC
21     D S S D G S E V K D V L L L D V T P L S

121    CTGGGTATCGAGACCAAGGGCGGGGTGATGACCAGGCTCATCGAGCGCAACACCACGATC
41     L G I E T K G G V M T R L I E R N T T I

181    CCCACCAAGCGGTCGGAGACTTTACCACCGCCGACGACAACCAACCGTCGGTGCAGATC
61     P T K R S E T F T T A D D N Q P S V Q I

241    CAGGTCTATCAGGGGGAGCGTGAGATCGCCGCGCACAACAAGTTGCTCGGGTCCTTCGAG
81     Q V Y Q G E R E I A A H N K L L G S F E

301    CTGACCGGCATCCCGCCGGCGCCGCGGGGATTCCGCAGATCGAGGCCACTTTCGACATC
101    L T G I P P A P R G I P Q I E A T F D I

361    GACGCCAACGGCATTGTGCACGTCACCGCCAAGGACAAGGGCACCGGCAAGGAGAGCACG
121    D A N G I V H V T A K D K G T G K E S T

421    ATCCGAATCCAGGAAGGCTCGGGCCTGTCCAAGGAAGACATTGACCGCATGATCAAGGAC
141    I R I Q E G S G L S K E D I D R M I K D

481    GCCGAAGCGCACGCCGAGGAGGATCGCAAGCGTCGCGAGGAGGCCGATGTTTCGTAATCAA
161    A E A H A E E D R K R R E E A D V R N Q

541    CCCGAGACATTGGTCTACCAGACGGAGAAGTTTCGTCAAAGAACAGCGTGAGGCCGAGGGT
181    A E T L V Y Q T E K F V K E Q R E A E G

601    GGTTCGAAGGTACCTGAAGACACGCTGAACAAGGTTGATGCCGCGGTGGCGGAAGCGAAG
201    G S K V P E D T L N K V D A A V A E A K

661    CCGGCACTTGGCGGATCGGATATTTTCGGCCATCAAGTCGGCGATGGAGAAGCTGGGCCAG
221    A A L G G S D I S A I K S A M E K L G Q

721    GAGTCGACGGCTCTGGGGCAAGCGATCTACGAAGCAGCTCAGGCTGCGTACAGGCCACT
241    E S Q A L G Q A I Y E A A Q A A S Q A T

781    GGCGTGCCACCCCGGCGGCGAGCCGGGCGGTGCCACCCCGGCTCGGCTGATGACGTT
261    G A A H P G G E P G G A H P G S A D D V

841    GTGGACGCGGAGGTGGTTCGACGACGGCCGGGAGGCCAAGTAG
281    V D A E V V D D G R E A K *

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Figure 5. Nucleotide sequence and derived amino acid sequence of fused M2e and HSP70₃₅₉₋₆₁₀. The M2e peptide and C-terminal domain of HSP70 (HSP70₃₅₉₋₆₁₀) are indicated in bold letters in italics, in which M2e is in black and HSP70₃₅₉₋₆₁₀ is in gray letters. The start and stop codons of ORF are indicated in gray boxes. The two amino acids related to *Bam*HI restriction enzyme is marked with rectangle and gray box.

Despite of these amino acids (aa 10-20) differences between human and non-human, it was reported that monoclonal antibodies (mAbs) recognizing the conserved SLLTEVET epitope of M2e potently inhibited influenza A virus replication in MDCK cells (Wang *et al* 2009), and passive transfer of a human mAb against M2e conserved sequence resulted in significant reduction in virus replication in the lung and protected mice from lethal infection (Wang *et al* 2008). Therefore, N-terminal epitope SLLTEVET, which is completely conserved among all hosts, could serve as an attractive target for development of universal influenza vaccines. One of the concerns about influenza A vaccine based on M2e protein is their limited potency; hence, optimal approaches to enhance immunogenicity of M2e protein immunization remain to be established. Up to now, several strategies have been applied to increase the potency of the vaccines based on small and low immunogen peptides, for example, targeting antigens to endoplasmic reticulum for rapid intracellular degradation (Hung *et al* 2001), directing antigens to APCs by fusion to ligands for APC receptors (Cusi *et al* 2004), co-injecting cytokines and co-stimulatory molecules (Calarota *et al* 2004). Expression of this fusion protein in *Pichia pastoris* yeast is underway, but further study will be needed to evaluate the adjuvant effect of the C-terminal domain of HSP70, HSP70₃₅₉₋₆₁₀, on extracellular domain of influenza A virus M2e protein and to explore the possible mechanisms and effectiveness of selected fragment of the HSP70 family in exerting adjuvanticity in a animal model.

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