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

Ebrahimi[*1], S.M., Tebianian[1], M., Mirjalili[1], A., Paykari[2], H., Varshovi[3], H.R., Toghyani[1], H., Moradi Bidhendi[4], S., Attaran[5], H.R.

1. Department of Biotechnology, Razi Vaccine & Serum Research Institute, Karaj, Iran
2. Department of Animal Viral Vaccine, Razi Vaccine & Serum Research Institute, Karaj, Iran
3. Department of Parasitology, Razi Vaccine & Serum Research Institute, Karaj, Iran
4. Department of Microbiology, Razi Vaccine & Serum Research Institute, Karaj, Iran
5. Department of Avian medicine, School of Veterinary Medicine, University of Shiraz, Shiraz, Iran

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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 ($HSP70_{359–610}$) 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 (hsp70 $_{359–610}$) 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

[*]Author for correspondence. E-mail: sm.ebrahimi@rvsri.ir

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 (Neirynck *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-teminal 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 $HSP70_{359\text{-}610}$- fused hepatitis B virus DNA vaccination resulted in a significant increase in hepatitis B surface antigen (HBsAg) - specific humoral response, while the $HSP70_{1\text{-}360}$- 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 (hsp70 $_{359–610}$) 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 ($HSP70_{359–610}$) 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 *Eco*RI and *Bam*HI 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 ($HSP70_{359\text{-}610}$) 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 *Bam*HI and *Xba*I 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 HSP70$_{359\text{-}610}$ as a single ORF. The amplified fragments, *M2e* and *HSP*70$_{359\text{-}610}$ 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*$_{359\text{-}610}$ 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$_{359\text{-}610}$ downstream primers. The PCR parameters were as above mentioned, but the melting temperature was 59 °C for 1 min.

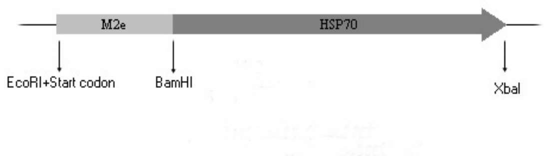

Figure 1 Schematic representation of the fused M2e and HSP70 $_{359\text{-}610}$ genes as a one open reading frame (~900-bp).

Construction of recombinant plasmid pPICMH. The PCR product containing both *M2e* and *HSP*$_{359\text{-}610}$ 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.

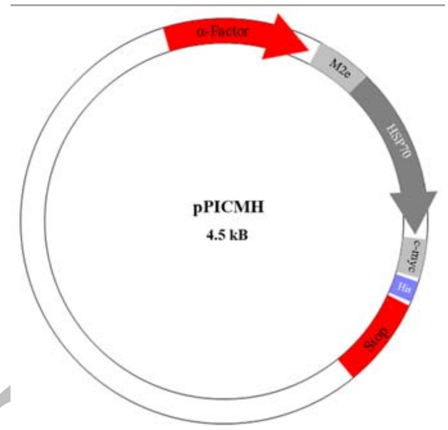

Figure 2. Schematic representation of the recombinant expression vector; pPICMH plasmid, that encoded *M2e-HSP70$_{359\text{-}610}$-Cmyc-His* fusion as a secretory protein based on the presence of α-factor signal sequence. P_{AOX1} denotes to the alcohol oxidase promoter and *Eco*RI, *Bam*HI and *Xba*I 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$_{359\text{-}610}$ (~820 bp: containing restriction site linker) genes, then the M2e gene was successfully ligated into the N-terminal of HSP70$_{359\text{-}610}$ to form an M2e- HSP70$_{359\text{-}610}$ 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$_{359\text{-}610}$ 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

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

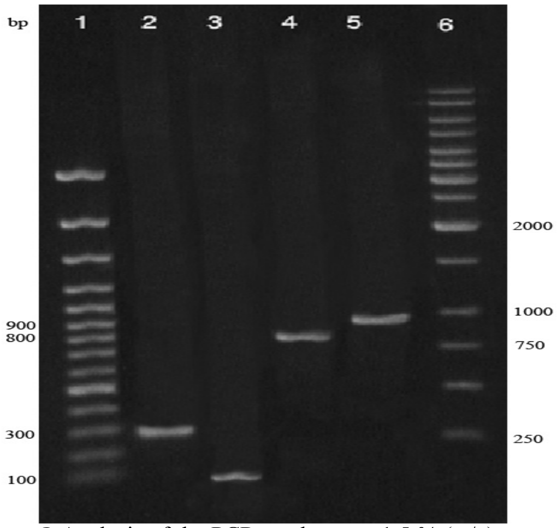

Figure 3 Analysis of the PCR products on 1.5 % (w/v) agarose gel. Lane 1, contains 100 bp ladder (Fermentas, Germany); lane 2, contains ~300 bp *M2* gene; lane 3, contains ~100 bp M2 extracellular domain (M2e); lane 4, contains ~820 bp $HSP70_{359-610}$; lane 5, contains ~900 bp M2e-$HSP70_{359-610}$ (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, Neirynck *et al* 1999, Tompkins *et al* 2007, Ernst *et al* 2006).

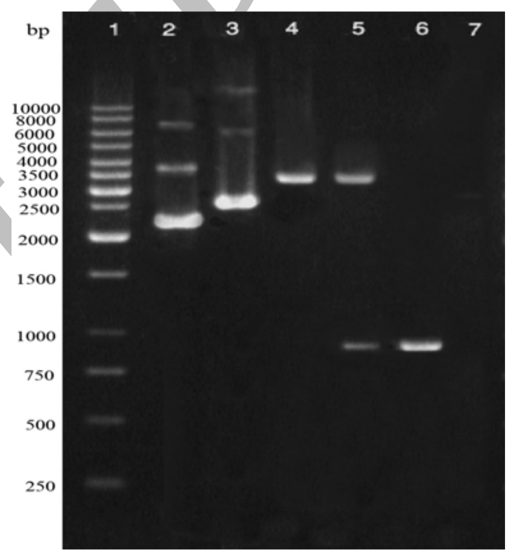

Figure 4. PCR and restriction enzyme analysis of the constructed pPICMH plasmid on 1.2 % (w/v) agarose gel. Lane 1: size marker 1 Kb (Fermentas, Lithuania); Lane 2: pPICZαA plasmid without insert; Lane 3: pPICZαA with insert (pPICZαA-M2e-$HSP70_{359-610}$, pPICMH); Lane 4: pPICZαA plasmid linerized by *Eco*RI enzyme; Lane 5: pPICMH double digested by *Eco*RI and *Xba*I 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 pPICZαA 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    CCCACCAAGCGGTCGGAGACTTTCACCACCGCCGACGACAACCAACCGTCGGTGCAGATC
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    CTGACCGGCATCCCGCCGGCGCCGCGGGGGATTCCGCAGATCGAGGCCACTTTCGACATC
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    GCCGAAGCGCACGCCGAGGAGGATCGCAAGCGTCGCGAGGAGGCCGATGTTCGTAATCAA
161     A  E  A  H  A  E  E  D  R  K  R  R  E  E  A  D  V  R  N  Q

541    GCCGAGACATTGGTCTACCAGACGGAGAAGTTCGTCAAAGAACAGCGTGAGGCCGAGGGT
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    GCGGCACTTGGCGGATCGGATATTTCGGCCATCAAGTCGGCGATGGAGAAGCTGGGCCAG
221     A  A  L  G  G  S  D  I  S  A  I  K  S  A  M  E  K  L  G  Q

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

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

841    GTGGACGCGGAGGTGGTCGACGACGGCCGGGAGGCCAAGTAG
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 $HSP70_{359\text{-}610}$. The M2e peptide and C-terminal domain of HSP70 ($HSP70_{359\text{-}610}$) are indicated in bold letters in italics, in which M2e is in black and $HSP70_{359\text{-}610}$ 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 serves 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, $HSP70_{359\text{-}610}$, 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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