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SEYED MAHMOUD EBRAHIMI

KHOSROW AGHAIYPOUR

HASSAN NILI

MAJID ESMAILZADEH

ALI MIRJALILI

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Construction of a pAED4-M2 vector for expressing avian influenza A (H9N2) virus M2 gene as a universal recombinant vaccine model

Seyyed Mahmoud EBRAHIMI[1,2,*], Khosrow AGHAIYPOUR[1], Hassan NILI[2],
Majid ESMAILZADEH[1], Ali MIRJALILI[1]

[1]Department of Biotechnology, Razi Vaccine and Serum Research Institute, Karaj, Tehran - IRAN

[2]Department of Avian Medicine, School of Veterinary Medicine, Shiraz University - IRAN

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Abstract: We expressed the M2 gene in prokaryotic cells using the pAED4 expression vector system to produce native and purified M2 protein as a candidate for universal recombinant vaccine against influenza A subtypes. The open reading frame (ORF) of avian influenza A/chicken/Iran/101/1998 (H9N2) M2 gene was amplified by 2-step RT-PCR using specific primers and pfu DNA polymerase. pAED4 was used as expression vector, purified PCR product digested by Nde I and EcoR I restriction enzymes was ligated to the same digested site in the vector using T4 ligase to form pAED4-M2.
The cloned M2 gene was confirmed by PCR and restriction enzymes pattern. M2 polypeptide was produced through the expression of this recombinant expression vector (pAED4+M2) in *Escherichia coli* BL21 (DE3) strain. The expressed M2 polypeptide was analyzed on SDS-PAGE and confirmed by western blotting assay. The level of 100% homology between the N-terminal domain of H5 and H9 isolates was considerable. It seems that recombinant vaccine based on Iranian isolate A/chicken/Iran/101/1998(H9N2) M2 protein might cover all H5 and H9 circulating in Iran and neighboring regions significantly. Further research will be needed to evaluate the immunity of the expressed M2 protein in the lab animal model to check the native conformational structure of this expressed protein by challenging with other influenza isolates.

Key words: M2 protein, pAED4, SDS-PAGE, expression, avian influenza

Introduction

Yearly development of influenza vaccines that are antigenically matched to circulating strains poses extraordinary challenges. A rapidly developing pandemic would shorten the time for strain identification and vaccine preparation; meanwhile, antigenic changes would continue. Moreover, the need to immunize an entirely naive population would exacerbate problems with vaccine production and supply. Vaccines based on conserved antigens would not require prediction of which strains would circulate during an approaching season and could avoid hurried manufacturing in response to

* E-mail: smebrahimi@shirazu.ac.ir

outbreaks. Test vaccination with DNA constructs that express conserved influenza A nucleoprotein (NP) or NP plus matrix (M) induced antibody and T-cell responses and protected against heterosubtypic viruses (1,2). Despite the virulence and rapid kinetics of challenge infection, DNA vaccination with NP and M achieved limited protection against an H5N1 virus strain isolated from the 1997 human outbreak in Hong Kong (3).

The gene segment 7 of avian influenza A encodes 2 proteins, both highly conserved: M1, the capside protein, and M2, an ion channel protein. M2 contains a small ectodomain (M2e), which makes it a target for antibody-based immunity. The ability of anti-M2 monoclonal antibody (MAb) to reduce viral replication (4) implicates M2, in particular M2e, as a vaccine target. M2 vaccine candidates that have been explored include peptide-carrier conjugates (5), baculovirus-expressed M2 (6), fusion proteins (7,8), multiple antigenic peptides (9), and M DNA constructs that potentially express M2 (10,11). Therefore, in this study we expressed the M2 gene in prokaryotic cells using the pAED4 expression vector system to produce native and purified M2 protein as a candidate for universal recombinant vaccine against influenza A subtypes.

Materials and methods

Viral isolate and viral RNA preparation: Avian influenza A/chicken/Iran/101/1998 (H9N2) used as a vaccinal seed in Marand branch of Iran's Razi Institute was grown in primary SPF chicken embryo fibroblast (CEF). Influenza virus infected cells were collected after 18 h by centrifugation at 3000 × g for 10 min following cell culture trypsinization.

Total RNA was extracted by the guanidinium isothiocyanate method using RNX-Plus™ (Tehran, Cinagen, Iran) solution. Then the extracted total RNA dissolved in 20 µL of RNase-free water (DEPC water).

Primer design: To clone full-length spliced M2 mRNA in cell culture, 2 gene-specific primers (M2U and M2L) were designed, and the gene specific sequences were selected by comparison and alignment of M2 gene sequences retrieved from the GenBank database by DNAMAN (version 4.13) and Oligo (version 5) software.

The M2U (Forward) primer: 5′GGAATTCCATATGAGTCTTCTAACCGAG 3′ contained an *Nde I* restriction site before the start codon, and the M2L (Reverse) primer: 5′GGAATTCCTTACTCCAGCTCTATGTTG 3′ contained an *EcoR I* restriction site after the stop codon.

cDNA synthesis and RT-PCR: After total RNA extraction, the cDNA synthesis was performed by AMV first strand cDNA synthesis kit (Mannheim, Roche, Germany) using random hexamer oligoes according to the manufacturer's protocol. Then the cDNA was amplified using specific primers (M2U and M2L) for the 310 bp fragment of the spliced M2 gene containing restriction site sequences.

A polymerase chain reaction (PCR) was carried out in a 50 µL mixture containing 5 µL of 10× reaction buffer with $MgSO_4$, 4 µL of mixed dNTPs (2.5 mM each), 1 µL of each specific primer (10 pmol each), 0.5 µL of pfu DNA polymerase (2.5 u/µL) (Mannheim, Roche, Germany), 3 µL of cDNA template, and 35.5 µL of DEPC water. The PCR program was 95 °C for 3 min, 5 cycles of 95 °C for 1 min, 54 °C for 1 min, and 72 °C for 1 min, then 30 cycles of 95 °C for 1 min, 56 °C for 1 min, and 72 °C for 1 min, followed by 72 °C for 10 min. At the end the PCR products were analyzed by 1.5% agarose electrophoresis.

Construction of expression vector: The PCR products were run on 1% agarose gel electrophoresis and the distinct band was purified using a PCR product purification kit (Mannheim, Roche, Germany) according to the manufacturer's protocol from gel for double digestion. pAED4 was used as expression vector, and purified PCR product digested by *Nde I* and *EcoR I* (Fermentas, Vilnius, Lithuania) restriction enzymes was ligated to the same digested site in the vector using T4 ligase (Fermentas, Vilnius, Lithuania) to form pAED4-M2

Expression of recombinant M2 protein: pAED4-M2 was transformed into *Escherichia coli* BL21 (DE3) competent cells. The cells were cultured in LB broth containing 100 µg/mL ampicillin and incubated at 37 °C on a shaker incubator until the optical density at

600 nm reached 0.6. Then 0.5 mM of isopropythio β-D-galactosidase (IPTG) was added to the medium to induce M2 gene expression. Samples were collected before (zero time) and after (1, 2, and 4 h) induction of expression for SDS-PAGE analysis.

SDS-PAGE and immunoblotting: For characterization of expressed M2 protein, samples were lysed by addition of protein sample buffer and heated at 100 °C for 5-10 min. Then extracts were electrophoresed in SDS-17.5% polyacrylamide gel and transferred into a polyvinylidene fluoride (PVDF) membrane (Roch, Germany). After blocking with 3% BSA, the immunoblotting was carried out with rabbit anti-influenza A virus M2 protein polyclonal Ab (abcam, Cambridge, UK) and mouse anti-rabbit IgG HRP (Santa Cruz, California, USA) according to the manufacturer's protocol.

Results

Analysis of the M2 gene sequences: PCR was able to amplify the desirable fragment (294 bp) of the open reading frame (ORF) of A/chicken/Iran/101/1998 (H9N2) spliced M2 gene (Figure 1). The maximum M2 gene homology was between the Iranian isolate and H9 and H5 isolates from Dubai, Pakistan, and Hong Kong. The nucleotide sequence of the Iranian isolate M2 gene was just 2% divergent from the most closely related viruses in GenBank. The nucleotides sequence of this gene was deposited in GenBank as accession number EU477375.

Gene analysis in vector: The desirable fragment of the M2 gene was cloned into the multiple cloning site region of the pAED4 expression vector. Identity of the cloned gene was confirmed by PCR and restriction analysis (Figure 2).

Gene expression and immunoblotting: A band with approximately 15 KDa of M2 protein was observed 1, 2, and 4 h after induction while this band was not seen in the negative control or positive control (Figure 3). The desirable band was confirmed by western blotting analysis using polyclonal M2 protein antibody (Figure 4).

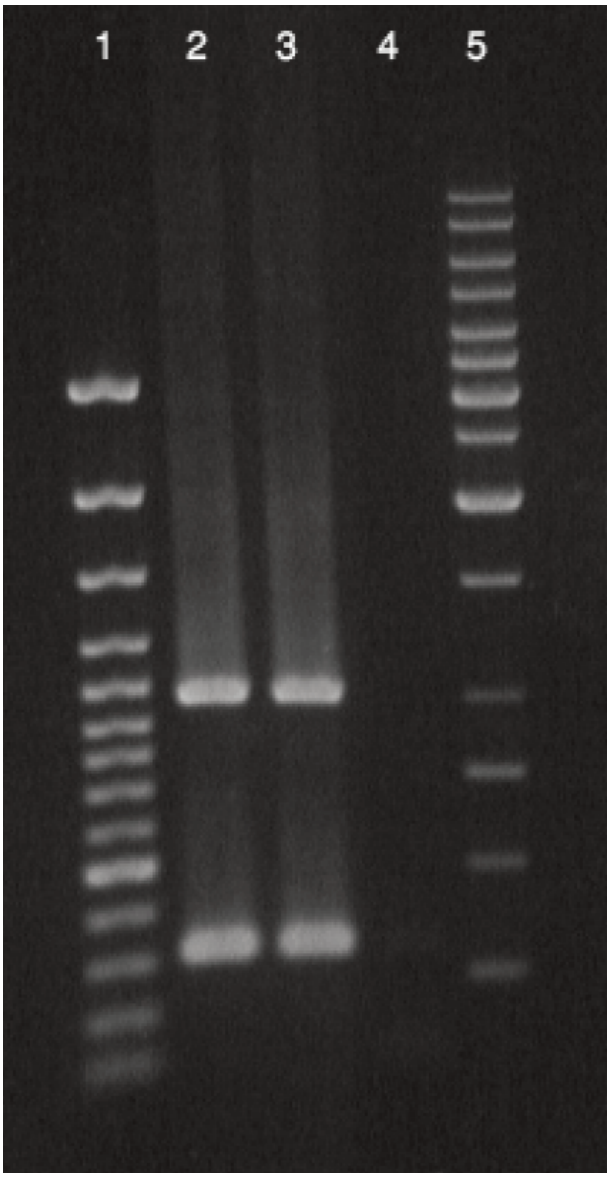

Figure 1. Analysis of PCR product on 1.2% agarose gel, lane 1, contains a 100-bp DNA ladder; lanes 2 and 3 contain positive samples in which the upper bands are segment 7 (26-1027 nt) and the lower bands are the M2 gene (ORF); lane 4 contains a negative control; lane 5 contains a 1 kb DNA ladder.

Discussion

The M2 protein is abundantly expressed at the surface of virus infected cells, but in comparison with the unstable hemagglutinin (HA) and neuraminidase (NA) glycoproteins, the M2 protein is a minor component of virions. It has essential role in the life

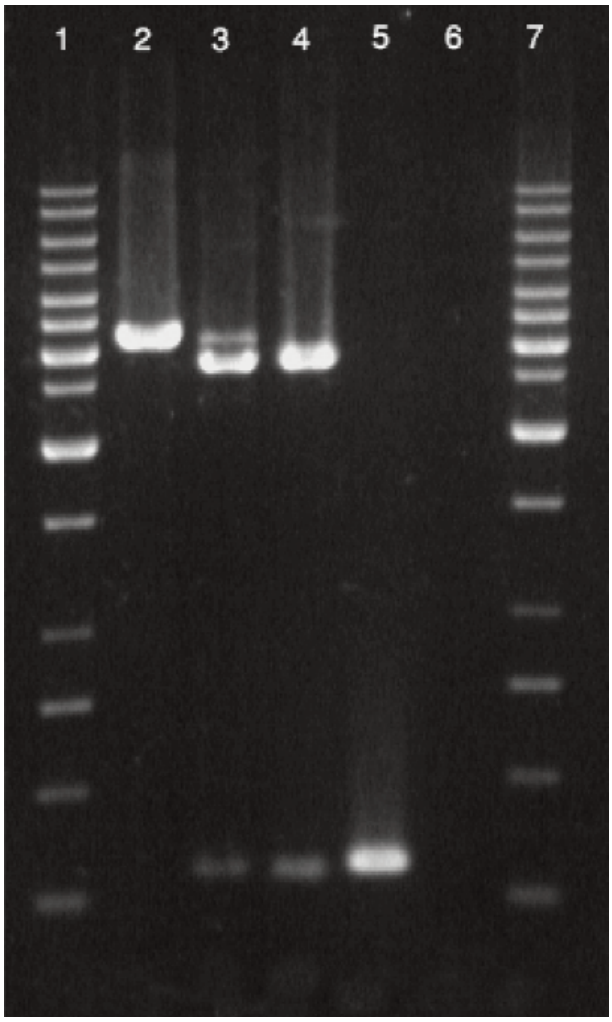

Figure 2. PCR and restriction enzyme analysis of the constructed pAED4+M2. Lane 1, 7: size marker 1Kb (fermentas); lane 2: pAED4 with insert (pAED4+M2); lane 3: pAED4+M2 product partially digested with Nde I and EcoR I; lane 4: pAED4+M2 product completely digested with Nde I and EcoR I; lane 5: PCR analysis of pAED4+M2 using specific primers; lane 6: negative control.

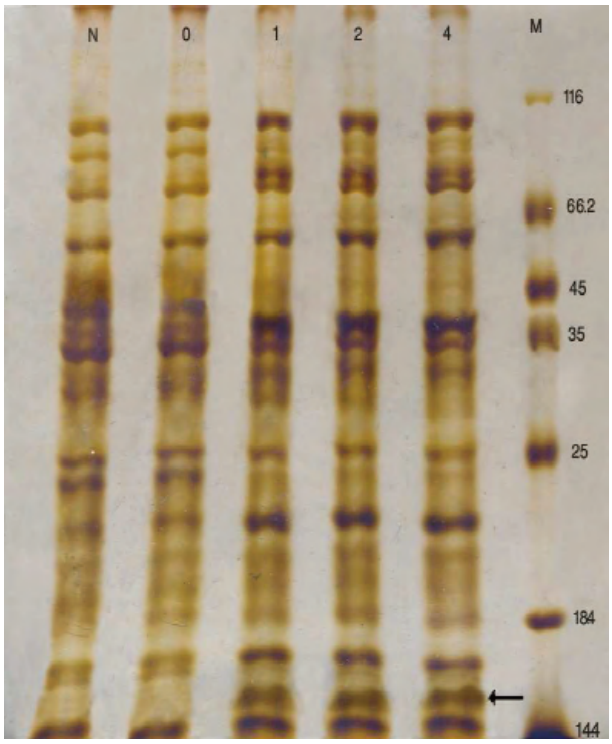

Figure 3. SDS-PAGE analysis of *E. coli* BL21 (DE3) stained by silver nitrate and showing the expression of the M2 gene. Lane M: negative control (bacteria without plasmid); lane 0: bacteria with plasmid before induction; lane 1, 2, 4: bacteria with expression plasmid 1, 2, 4 h after induction, respectively; lane M: low molecular size marker (Fermentas).

cycle of influenza virus replication. The current strategy for developing vaccine has been focused on inducing neutralizing antibody against HA or NA glycoproteins, but it was indicated that these 2 glycoproteins show great antigenic diversity among influenza A subtypes (12).

Extracellular M2 protein can be suggested as a target of host humoral immunity induction. Therefore, it can produce antibody as an inhibitory antibody against influenza A subtypes by blocking the extracellular domain of M2 homotetramer protein. This way of blocking can inhibit viral fusion and budding of viral particles after viral host cell entry. This inhibition of viral replication is more efficient than the amantadine and remantadine antiviral drugs, which block the viral replication by blocking the transmembrane domain of M2 ion channel protein on virus particles and apical surface of virus-infected cells. Previous research has shown that an anti-M2 protein antibody can restrict virus growth in vitro (4) and partially protect passively immunized mice from infection with influenza virus (13).

Our sequence alignment showed that the M2 protein from avian influenza A virus strain is conserved among all isolates from different hosts and areas, and the N-terminal domain of the M2 protein,

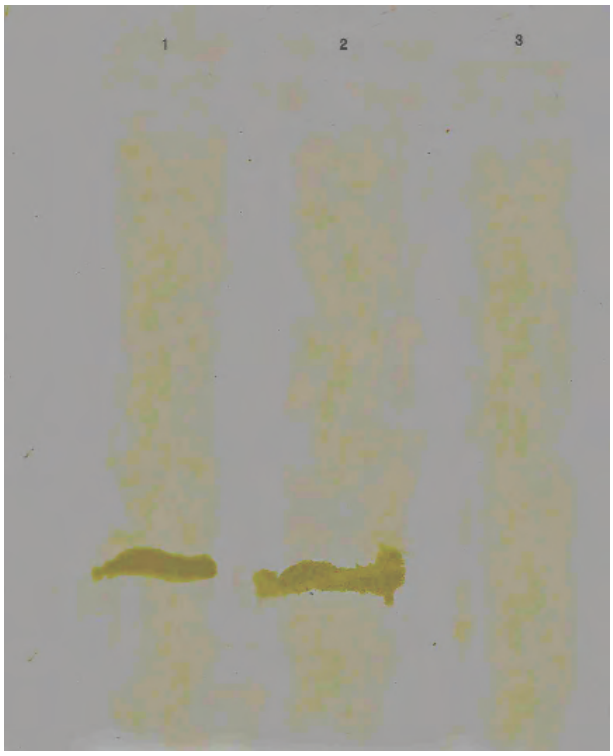

Figure 4. Western blot analysis of expressed M2 protein on PVDF membrane using rabbit anti-influenza A virus M2 protein polyclonal Ab (abcam, UK) and mouse anti-rabbit IgG HRP (Santa Cruz, California, USA). Lane 1, 2: positive reaction; lane 3: negative control.

which is the immunogenic portion, is highly conserved among H9 and H5 isolates deposited in GenBank.

In the present study, we used pAED4 expression vector to produce M2 protein in a native form. Western blotting assay with M2 protein polyclonal antibody induced by H2N2 human origin (strain A/Ann Arbor/6/60 [H2N2]) reacted well with M2 protein expressed in this study. This cross-reactivity in addition to extraplotting sequence alignment could be a good indication that immunity against M2 protein of H9N2 might cover other influenza isolates, particularly H5N1.

We expressed M2 protein as a cytoplasmic inclusion body in the native and inactive form, western blotting or ELISA test can change the protein's conformational structure to linear form, and consequently can change the epitopes. Therefore, it might not be a good representative of native epitopes in influenza infected host cells.

Further research will be needed to evaluate the immunity of the expressed M2 protein in the lab animal model to check the native conformational structure of this expressed protein by challenging with other influenza isolates.

We cloned, sequenced, and expressed A/chicken/Iran/101/1998 (H9N2) in prokaryotic cells using the pAED4 expression system and the expressed M2 protein was confirmed by SDS-PAGE and western blotting assay. It seems that the recombinant vaccine based on Iranian isolate M2 protein might cover all H5 and H9 circulating in Iran and neighboring regions significantly.

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