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Prokaryotic expression and characterization of avian influenza A virus *M2* gene as a candidate for universal recombinant vaccine against influenza A subtypes; specially H5N1 and H9N2

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Abstract The conserved M2 protein of influenza A virus is considered as a promising candidate target for a broad-spectrum, recombinant influenza A vaccine. In the present study, the open reading frame (ORF) of avian influenza A/chicken/Iran/101/1998 (H9N2) *M2* gene was amplified then cloned in pAED4, prokaryotic expression vector. M2 protein was produced through the expression of this recombinant expression vector (pAED4-M2) in *E. coli* BL21 (DE3) strain. The expressed M2 protein was analyzed on SDS-PAGE. Western blot assay was used to examine the immunoreaction of the expressed protein using commercial polyclonal anti-M2 antibody. The antigenicity and biological activity of the recombinant protein was also qualitatively detected on infected MDCK cells surface by immunofluorescence assay using rabbit's immunized antiserum. So, according to the sequence alignment based on the mentioned isolate and the result of immunoassay reaction, it seems recombinant vaccine based on A/chicken/Iran/101/1998 (H9N2) M2 protein isolate might cover majority of influenza A virus strains specially H5 and H9 circulating in Iran and neighbor regions significantly.

Keywords M2 protein · pAED4 · SDS-PAGE · Expression · Avian influenza

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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 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 two proteins, both highly conserved: M1, the capsid 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, implicates M2, in particular M2e, as a vaccine target [4, 5]. M2 vaccine candidates that have been explored include peptide-carrier conjugates [6], baculovirus-expressed M2 [7], fusion proteins [8, 9], multiple antigenic peptides [10], and M DNA constructs that potentially express M2 [11, 12]. Therefore, in this study we expressed *M2* gene in prokaryotic cells using 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) using 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.

Primer design

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

The M2U (Forward) primer: 5′-GGAATTCCATATGA GTCTTCTAACCGAG-3′ contained *Nde*I restriction site before start codon, and the M2L (Reverse) primer: 5′-GGA ATTCCTTACTCCAGCTCTATGTTG-3′ contained *Eco*RI restriction site after 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 manufacture 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.

Polymerase chain reaction (PCR) was carried out in 50 μl mixture containing 5 μl of 10× reaction buffer with MgSO4, 4 μl of mixed dNTPs (2.5 mM each), 1 μl of each specific primers (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 nuclease-free 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, following by 72°C for 10 min in 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 from gel for double digestion using PCR product purification kit (Mannheim, Roche, Germany) according to manufacturer's protocol. The purified PCR product digested by *Nde*I and *Eco*RI (Fermentas, Vilnius, Lithuania) restriction enzymes was ligated to the same digested site in the pAED4 expression vector using *T4* ligase (Fermentas, Vilnius, Lithuania) to form recombinant expression vector (pAED4-M2).

Expression of recombinant M2 protein

pAED4-M2 was transformed into *E. 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 till 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 times) induction of expression for SDS-PAGE analysis.

SDS-PAGE and immunoblotting

For confirmation of the expressed 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 carried out with rabbit anti-influenza A virus M2 protein polyclonal Ab (Abcam, Cambridge, UK) and mouse anti-rabbit IgG HRP (Santu Cruz, California, USA) according to manufacturer's protocol.

Generation of polyclonal antiserum

Polyclonal antiserum to expressed M2 protein was prepared by immunizing rabbits with purified recombinant M2 protein emulsified in incomplete freund's adjuvant (IFA), then heated for 30 min at 57°C to inactivate complement and stored at −20°C [13].

Rabbit polyclonal anti-M2 antibody from A/Ann Arbor/6/60 (H2N2) origin and FITC-labeled goat anti-rabbit IgG were purchased from Abcam Co. (Abcam, Cambridge, UK).

Indirect immunofluorescence

Six-well plates were seeded with 10^7 Madin-Darby canine kidney (MDCK) cells per well. When 100% confluence of the MDCK cells was reached, the wells were infected (or left uninfected as a control) with 10 multiplicity 50% egg-

infective doses (EID_{50}) of influenza A virus isolate of A/chicken/Iran/101/1998 (H9N2) in a volume of 10 ml of DMEM (5% FCS, glutamine, and antibiotics) in a falcon tissue culture flask (75 cm^2; BD Biosciences, Franklin Lakes, NJ) and incubated at 37°C.

For surface staining, indirect immunofluorescence microscopy was carried out. Briefly, at 6 h postinfection (PI) cells were washed with PBS, fixed in 4% paraformaldehyde for 30 min at room temperature and then permeabilized in 0.5% Triton X-100 for 20 min at room temperature. Cells were washed with PBS, and the Influenza A virus matrix protein (M2) on MDCK cells surface were detected by using rabbit polyclonal anti-M2 antibody from A/Ann Arbor/6/60 (H2N2) origin (Abcam, Cambridge, UK), diluted 1:1,000 in PBS and antiserum from rabbit immunized with expressed M2 protein, diluted 1:250 in PBS for 1 h at 37°C.

The antibodies were removed and the cells were washed three times with PBS, after which they were incubated with the FITC-labeled goat anti-rabbit IgG (Abcam, Cambridge, UK) for 20 min at 37°C. The cells were washed three times with PBS and fluorescence was visualized by Nikon FXL microscope.

Results

Analysis of the *M2* gene sequences in vector

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. The desirable fragment of *M2* gene was cloned into the multiple coning site region of pAED4 expression vector (Fig. 1a).

Identity of the cloned gene was confirmed by PCR, restriction analysis and gene sequencing.

The nucleotide sequence of the Iranian isolate *M2* gene was just 2% divergent from the most closely related viruses in GenBank. At the nucleotide level based on *M2* gene, Iranian isolate was more closely related to its neighbor states like Dubai and Pakistan. Similar relationship could be seen between Iranian isolate and Hong Kong H9 and H5 isolates, with nucleotide sequence differences of about 2% and amino acid sequence homology of 100%. Nucleotides similarity seen between H5 and H9 isolates was more than other isolates from all hosts in different areas (data not shown).

The nucleotides sequence of this gene was deposited in GenBank under accession number EU477375.

Gene expression and immunoblotting

A band with approximately 15 kDa of M2 protein was observed in 1, 2, and 4 h after induction while this band was not seen in negative control and positive control (Fig. 1b). The desirable band was confirmed by Western blot analysis using polyclonal M2 protein antibody (Fig. 2).

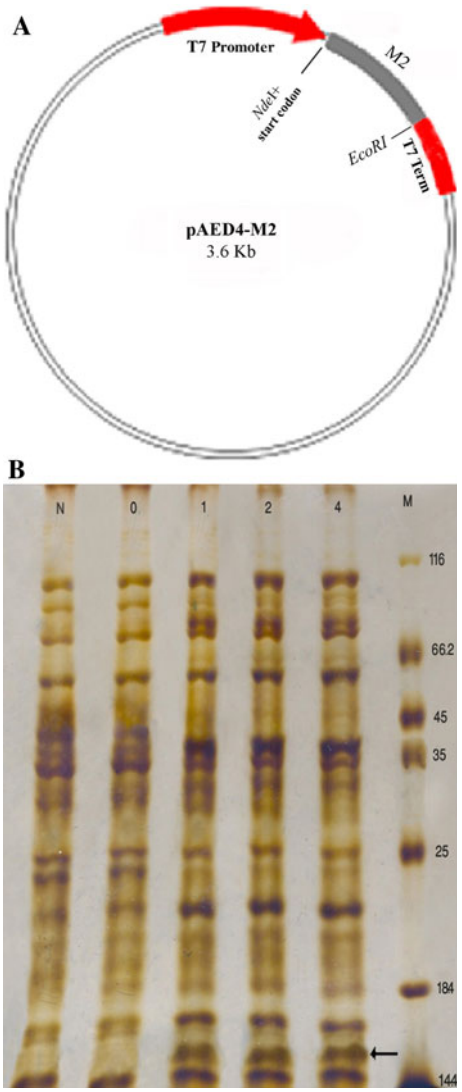

Fig. 1 **a** Schematic representation of the recombinant expression vector; plasmid AED4-M2 was constructed to express recombinant M2 protein. **b** SDS-PAGE analysis of *E. coli* BL21 (DE3) stained by Silver nitrate and showing the expression of M2 gene; *lane N* negative control (bacteria without plasmid); *lane 0* (zero) bacteria with plasmid before induction; *lane 1, 2, and 4* bacteria with expression plasmid 1, 2, and 4 h after induction, respectively; *lane M* low molecular size marker (Fermentas, Germany)

Indirect immunofluorescence assay

MDCK cells infected with mentioned isolate of avian influenza A virus (H9N2) was qualitatively analyzed by fluorescence microscopy using FITC-label goat anti-rabbit IgG antibody to evaluate the biological activity of the expressed M2 protein.

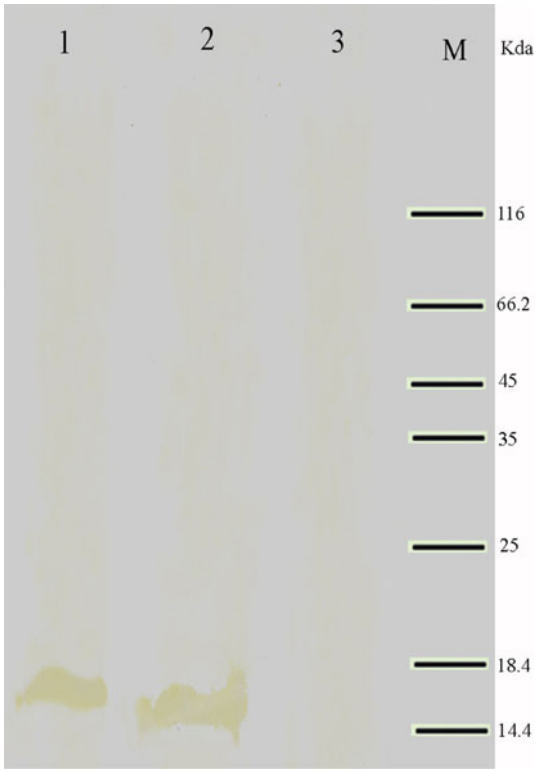

Fig. 2 Western blot analysis of expressed M2 protein on PVDF membrane using rabbit anti-influenza A virus M2 protein polyclonal Ab (Abcam, UK) and mouse anti-rabbit IgG HRP (Santu Cruz, California, USA). *Lane 1, 2* positive reaction; *lane 3* negative control, *lane M* schematic representation of low molecular size marker (Fermentas, Germany)

Uninfected cells were used as negative control and cells stained with antiserum from rabbit immunized with expressed M2 protein did not show difference in fluorescence. As expected, cells infected with isolate of avian influenza virus showed a strong increase in green fluorescence (Fig. 3).

Discussion

There has been growing interest in M2 as a "universal" vaccine that may protect against a much wider range of avian influenza viruses (AIVs) than current vaccines. The potential of M2 as "universal" vaccine derives from the following observations: First, antibodies (Abs) directed against its ectodomain (M2e) have been shown to restrict virus replication and reduce severity of disease in animal models [9, 14]. Second, M2e shows a remarkably high degree of structural conservation amongst avian influenza A strains. Third, hosts currently appear to lack M2e-specific Ab-mediated protection. This has been indicated by two studies that measured M2 specific Ab titers in human

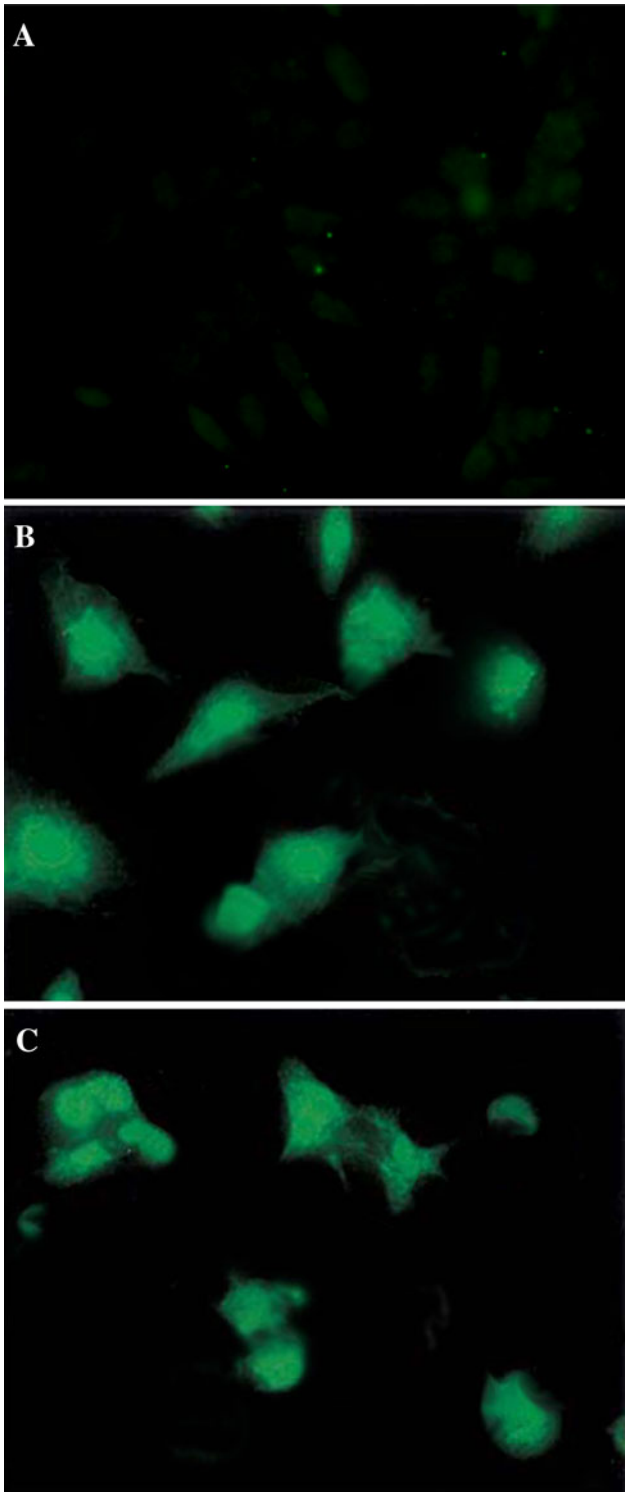

Fig. 3 Cells were not infected by influenza virus using lab produced rabbit antiserum for immunofluorescence analysis (**a**); cells were infected with influenza A virus A/chicken/Iran/101/1998 (H9N2) isolate for 6 h and analyzed by immunofluorescence microscopy using lab produced rabbit antiserum (**b**) and commercial anti-M2 antibodies (**c**), respectively

sera [15, 16]. These studies indicated that M2-specific Ab responses were inconsistently and poorly induced in humans by influenza A virus infection and, if induced, appeared to be of low titer and short duration. Similar findings were made in mice, in which recovery from pulmonary infection also did not result in substantial M2e-specific Ab titers when measured by ELISA against M2e peptide [6, 17]. Taken together, the results confirmed the generally poor induction of the M2e-specific Ab response by avian influenza A virus infection and indicated that hosts currently lack optimal M2e-specific protection. Anti-M2 antibody despite of blocking the channel activity of influenza A virus particle or infected cell, it can also induce indirect mechanism of protection such as complement-mediated cytotoxicity (CDC) or Ab-dependent cytotoxicity (ADCC). So, this provides an important reason for development of an M2-specific vaccine [18].

Our previous study showed that the N-terminal domain of the M2 protein was highly conserved among all H5 and H9 subtypes isolated in avian, swine, equine and human of Influenza A viruses in different areas. Thus, immunity against M2e protein of H9N2 might cover H9 and H5 isolates of influenza A more significantly than others, especially those H5 and H9 circulating in Middle East and Eastern states of Asia [5].

Therefore, in this study, the project of influenza A (H9N2) virus *M2* gene expression as a candidate for recombinant vaccine against H5 and H9 subtypes was carried out and pAED4 expression vector was used to produce M2 protein in a native form. The expressed M2 protein showed to have an apparent $M_r \approx 15$ kDa on polyacrylamide gel, but; the sequence indicates it contains only 97 amino acids. Thus, the migration of the M2 polypeptide on polyacrylamide gel is anomalous with respect to size and depends on amino acids composition; these results are consistent with those described in a previous report [19, 20]. Western blot analysis with commercial rabbit anti-M2 polyclonal antibody induced by H2N2 human origin (strain A/Ann Arbor/6/60 [H2N2]) showed well-reacted with expressed M2 protein.

Moreover, the result of indirect immunofluorescence assay indicated that the expressed M2 protein is not only biologically active, but also could detect two different strains of avian influenza virus (H9N2) and influenza virus (H2N2).

So, this allows two conclusions. First, Abs induced against M2 peptide are cross-reactive with a protein expressed on virus-infected cells, apparently native M2 protein, and second, Abs probably act in vivo by binding to the surface of infected cells. Thus, protection is probably dependent on Fc- or complement- mediated effector function such as ADCC or CDC.

In conclusion, the M2 protein was successfully expressed in prokaryotic system and biological activity of which was confirmed.

According to the sequence alignment based on the mentioned isolate and the result of immunoassay reaction, it seems recombinant vaccine based on A/chicken/Iran/101/1998(H9N2) M2 protein isolate might cover majority of influenza A virus strains specially H5 and H9 circulating in Iran and neighbor regions significantly.

In conclusion, for presenting M2 protein as a universal vaccine candidate against influenza A virus, further study seems to be necessary to evaluate the immunity of this protein in the lab animal by challenging with other influenza A isolates.

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