Short Communication

Sequence analysis of *M2* gene of avian influenza virus strain (A/Chicken/Iran/101/98 (H9N2)) as an oil vaccine seed

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Abstract
In this study, the full-length *M2* gene of the avian influenza virus (H9N2) was isolated, analyzed and studied in detail. Total RNA was extracted and cDNA of the M2 mRNA was obtained by reverse transcriptase polymerase chain reaction (RT-PCR) using random hexamer oligoes; specific primers were used for amplification of the M2 open reading frame (ORF) region. PCR was able to amplify the desirable fragment (294-bp) of the spliced *M2* gene. The nucleotide sequence homology between the Iranian isolate and other H9 and H5 subtypes of influenza A from different hosts and geographical areas deposited in GenBank ranged from 92 to 98% and the amino acid sequence homology ranged from 97 to 100%.
Keywords: *M2* gene; Sequence; RT-PCR; H9N2.

Influenza A virus expresses two highly immunogenic, but variable, transmembrane proteins; hemagglutinin (HA) and Neuraminidase (NA) which constantly evolve by the mechanisms of antigenic drift and antigenic shift (Webster *et al*., 1992). Another transmembrane protein of the influenza A virus is an M2 integral membrane protein. A large number of M2 molecules are expressed at the plasma membrane of the influenza virus-infected cell surface, with a ratio of approximately two M2 molecules per HA (Zebedee *et al*., 1988). The 97-amino acid M2 protein is a homotetrameric integral membrane protein that exhibits ion channel activity and is composed of 24 extracellular amino acids, 19 transmembrane amino acids, and 54 cytoplasmic residues (Bauer *et al*., 1999; Holsinger *et al*., 1991; Lamb *et al*., 1985). Disulfide bonds link the protein through cysteines located in the extracellular region (Holsinger *et al*., 1991); the protein (ion channel) is the target of antiviral drugs like amantadine and remantadine. The ion channel activity of M2 is important both during virion uncoating and during viral budding. Certain mutations in the *M2* gene lead to viruses that are resistant to antiviral drugs (Pinto *et al*., 1992; Hay *et al*., 1985).

In recent years scientists have focused on the influenza M2 protein as a candidate for a universal vaccine. The present study involved the analysis of the M2 open reading frame (ORF) of the A/chicken/Iran/101/1998 avian influenza (H9N2) isolate using as an inactivated vaccine seed from the Razi Vaccine and Serum Research Institute in order to evaluate the homology level between this isolate and others deposited in GenBank.

For this purpose the avian influenza A/chicken/Iran/101/1998 (H9N2) used as a vaccinal seed in the Marand branch of Iran's Razi Institute was grown in primary chicken embryo cell cultures and cells were maintained in a humidified air-5% CO_2 atmosphere at 37ºC. Then the Influenza virus infected cells were then collected after 18 h of incubation by centrifugation at 3000 $\times g$ for 10 min following cell culture tripsinization.

Total RNA was extracted by the guanidinium isoth-

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iocyanate method using RNX-Plus™ (Cinagen, Tehran, Iran) solution. The extracted total RNA was then dissolved in 20 µl of RNase-free water. 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 the *M2* gene sequences retrieved from the GenBank database by DNAMAN (version 4.13) and Oligo (version 5) software. The designed primers sequences are as follows: M2U (Forward): 5′GGAATTCCATATGAGTCTTCTAACCGAG3′ and the M2L (Reverse): 5′GGAATTCCTTACTCCAGCTCTATGTTG 3′.

After RNA extraction, cDNA synthesis was prepared by the AMV first strand cDNA synthesis kit (Mannheim, Roche, Germany) using random hexamer oligoes, according to the manufacture's protocol. The cDNA was then amplified using specific primers (M2U and M2L) resulting in a 310 bp fragment of the spliced *M2* gene containing restriction enzymes site sequences.

Polymerase chain reaction (PCR) was performed in a 50 µl mixture containing 5 µl of 10X reaction buffer with $MgSO_4$ (2mM), 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 involved denaturation at 95ºC for 3 min, followed by 5 cycles of denaturation at 95ºC for 1 min, annealing at 54ºC for 1 min, extension at 72ºC for 1 min, and then 30 cycles of denaturation at 95°C for 1 min, annealing at 56ºC for 1 min, extension at 72ºC for 1 min, followed by a final extension at 72ºC for 10 min. The resulting PCR products were then analyzed by 1.5% (w/v) agarose gel electrophoresis. The PCR products were subsequently run on a 1% (w/v) agarose gel and the distinct band was purified using PCR product purification kit (Mannheim, Roche, Germany), according to manufacture protocol. The resulting purified product was sequenced by the MWG Biotech Co. (Germany). All sequence data were identified by Blast search in the NCBI database and compared with registered avian influenza isolates in GenBank.

The PCR technique was able to amplify two distinct bands, the desirable fragment (310 bp) of the spliced *M2* gene ORF containing restriction enzymes site sequences and the fragment (1000 pb) of the segment 7 gene belonging to the A/chicken/Iran/101/1998 (H9N2) (Fig. 1). The nucleotide

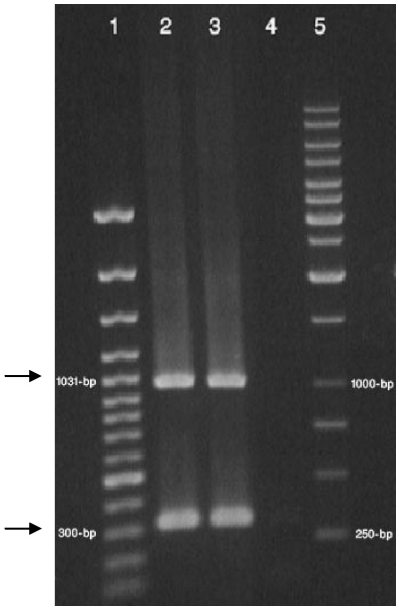

Figure 1. Analysis of the PCR product on 1.2 % (w/v) agarose gel. Lane 1, contains a 100-bp DNA ladder (Fermentas, Germany); lane 2 and 3, contain positive samples with the upper bands representing segment 7 (26-1027 nt) and the lower bands representingthe *M2* gene (ORF); lane 4, contains a negative control; lane 5, contains a 1kb DNA ladder (Fermentas, Germany).

sequence homology between the Iranian isolate and the other GenBank deposited isolates of influenza A viruses from different hosts and geographical areas ranged from 87% to 98% (data not shown). The maximum *M2* gene homology was observed between the Iranian isolate and the H9 and H5 isolates from Dubai, Pakistan, and Hong Kong states (Fig. 2).

To perform a direct comparison between the Iranian M2 extracellular domain and those of the other isolates deposited in GenBank, the amino acid sequences of all isolates were compared with that of the A/chicken/Iran/101/1998 (H9N2) representing as a baseline sequence. The amino acid sequence homologies ranged from 92% to 100% among all H5 and H9 isolates of influenza A obtained from different hosts and geographical areas (Fig. 3).

The nucleotide sequence of the Iranian *M2* gene isolate diverged by 2% from the most closely related viruses in GenBank. At the nucleotide level based on the *M2* gene, the Iranian isolate was more closely related to its neighboring states, such as Dubai and Pakistan. A similar relationship could be seen between the Iranian isolate and the Hong Kong H9 and H5 isolates, exhibiting nucleotide sequence differences of approximately 2% and amino acid sequence homology of 100%. Nucleotides similarities observed between

the H5 and H9 isolates were more than other isolates that were derived from hosts located in different areas. The N-terminal domain of the influenza A M2 protein is the major part of this immunogenic protein (Neirynck *et al*., 1999). Hence, in this study the *M2* open reading frame was translated and the region between amino acids position 1 and 24 of the M2 protein was analyzed. Sequence alignment based on the amino acid sequences of the M2 protein exhibited less diversity than the nucleotide-based sequence alignments. The 100% homology level between the N-terminal domain of the H5 and H9 isolates was considerable. Those which did not show 100% homology only possessed substitution of 1 and rarely 2 residues within residue 10 and 16 over a span of 24 residues. These changes are consistent with those described in previous reports (Widjaja *et al*., 2004).

Meanwhile, comparisons of the amino acid sequences of the M2 protein belonging to the Iranian H9N2 isolate with all other isolates of influenza A deposited in GenBank, indicated that the gene and amino acid sequences between all H9 and H5 isolates are more closely related than others. In this study, from the alignment of the *M2* gene and comparison of amino acids, it was not possible to find an *M2* gene or amino acid substitution that was a host or region-related substitution. They tend to vary more between species than within them and do not appear to change progressively over time, such as the surface glycoprotiens.

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A/chicken/Iran/101/1998(H9N2)   ATGAGTCTTCTAACCGAGGTCGAAACGCTTACCAGAAACG     40
A/chicken/Dubai/463/2003(H9N2) ----------------------------------------     40
A/chicken/Pakistan/2/99(H9N2)   -----C----------------------------------     40
A/Hong Kong/1073/99(H9N2)       ----------------------------------------     40
A/Hong Kong/532/1997(H5N1)      ----------------------------------------     40

A/chicken/Iran/101/1998(H9N2)   GATGGGGTTGCAGATGCAGCGATTCAAGTGATCCTCTCGT     80
A/chicken/Dubai/463/2003(H9N2) ----------------------------------------     80
A/chickenPakistan/2/99(H9N2)    ------AG----------------------------------     80
A/Hong Kong/1073/99(H9N2)       ------AG----A------A--------------------     80
A/Hong Kong/5321997(H5N1)       -------g---------------------------------     80

A/chickenIran101/1998(H9N2)     TGTTGCAGCAAGTATCATTGGGATATTGCACTTGATATTG    120
A/chicken/Dubai/463/2003(H9N2) -----------A----------------------------    120
A/chicken/Pakistan/2/99(H9N2)  ----------------------------------------    120
A/Hong Kong/1073/99(H9N2)       ----------------------------------------    120
A/Hong Kong/532/1997(H5N1)      ----------------------------------------    120

A/chicken/Iran/101/1998(H9N2)   TGGATTCTTGATCGTCTTTTTTTCAAATGCATTTATCGTC    160
A/chicken/Dubai/463/2003(H9N2) ----------------------------------------    160
A/chicken/Pakistan/2/99(H9N2)  --------------------C-------------------    160
A/Hong Kong/1073/99(H9N2)       --------------------C-------------------    160
A/Hong Kong/532/1997(H5N1)      --------------------C-------------------    160

A/chicken/Iran/101/1998(H9N2)   GCTTTAAATACGGTTTGAAAAGAGGGCCTTCTACGGAAGG    200
A/chicken/Dubai/463/2003(H9N2) ----------------------------------------    200
A/chicken/Pakistan/2/99(H9N2)  ----------------------------------------    200
A/Hong Kong/1073/99(H9N2)       ----------------------------------------    200
A/Hong Kong/532/1997(H5N1)      ----------------------------------------    200

A/chicken/Iran/101/1998(H9N2)   AGTACCTGAGTCTATGAGGGAAGAGTATCGGCAGGAACAG    240
A/chicken/Dubai/463/2003(H9N2) ---G------------------------------G---    240
A/chicken/Pakistan/2/99(H9N2)  ---G-------------------------------------    240
A/Hong Kong/1073/99(H9N2)       ---G-------------------------------------    240
A/Hong Kong/532/1997(H5N1)      ---G-------------------------------------    240

A/chicken/Iran/101/1998(H9N2)   CAGAGTGCTGTGGATGTTGACGATGGTCATTTTGTCAACA    280
A/chicken/Dubai/463/2003(H9N2) ----------------------------------------    280
A/chicken/Pakistan/2/99(H9N2)  ----A-----------------------------------    280
A/Hong Kong/1073/99(H9N2)       ----A-----------------------------------    280
A/Hong Kong/532/1997(H5N1)      ----A-----------------------------------    280

A/chicken/Iran/101/1998(H9N2)   TAGAGCTGGAGTAA                            294
A/chicken/Dubai/463/2003(H9N2) --------------                            294
A/chicken/Pakistan/2/99(H9N2)  --------------                            294
A/Hong Kong/1073/99(H9N2)       --------------                            294
A/Hong Kong/532/1997(H5N1)      --------------                            294
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Figure 2. Alignment of the *M2* gene sequences. Nucleotides matching those of the A/chicken/Iran/101/1998(H9N2) are shown as dashes.

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A/chicken/Iran/101/1998(H9N2)          MSLLTEVETLTRNGWGCRCSDSSD     24
A/partridge/Shantou/24/2000(H9N2)      ------------------------     24
A/chicken/Dubai/463/2003(H9N2)         ------------------------     24
A/quail/Dubai/301/2000(H9N2)           ------------------------     24
A/Quail/Hong Kong/G1/97 (H9N2)         ------------------------     24
A/Hong Kong/491/97(H5N1)               ------------------------     24

A/chicken/Shantou/4726/2004(H9N2       ---------Y--------------     24

A/Guinea fowl/Shantou/1677/2000(H9N2)  ---------H--------------     24
A/duck/Shantou/3460/2003(H9N2)         ---------H--------------     24
A/chicken/Yunnan/nh/2001(H9N2)         ---------H--------------     24

A/duck/Jiangsu/nf/2002(H9N2)           ---------P--------------     24
A/swine/Shandong/na/2003(H9N2)         ---------P--------------     24

A/duck/Viet Nam/Ncvd1/2002(H5N1)       ---------P-----E--------     24
A/curlew/Shandong/61/04(H5N1)          ---------P-----E--------     24
A/chicken/Jilin/hj/2003(H5N1)          ---------P-----E--------     24
A/chicken/Hubei/wf/2002(H5N1)          ---------P-----E--------     24
A/chicken/Scotland/59(H5N1)            ---------P-----E--------     24
A/chicken/Jilin/9/2004(H5N1)           ---------P-----E--------     24
A/duck/Hunan/114/05(H5N1)              ---------P-----E--------     24
A/fowl/Weybridge(H7N7)                 ---------P-----E--------     24
A/chicken/Brescia/1902(H7N7)           ---------P-----E--------     24

A/mallard/Italy/299/05(H7N7)           ---------P-----E-K------     24
A/duck/Jiangxi/1760/03(H7N7)           ---------P-----E-K------     24
A/mallard/Italy/250/02(H7N1)           ---------P-----E-K------     24

A/turkey/Italy/68819/03(H7N3)          ---------PI----E--------     24
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Figure 3. Alignment of the M2 extra cellular domain protein sequences, the first 24 residues of the M2 protein are shown. Amino acids matching A/chicken/Iran/101/1998(H9N2) are shown as dashes.

Acknowledgments

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