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***Full Article***

# Sequence Analysis and Phylogenetic Profiling of the Nonstructural (NS) Genes of H9N2 Influenza A Viruses Isolated in Iran during 1998-2007

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**ABSTRACT**

The earliest evidences on circulation of Avian Influenza (AI) virus on the Iranian poultry farms date back to 1998. Great economic losses through dramatic drop in egg production and high mortality rates are characteristically attributed to H9N2 AI virus. In the present work non-structural (NS) genes of 10 Iranian H9N2 chicken AI viruses collected during 1998-2007 were fully sequenced and subjected to a phylogenetic analysis. The observations proved allele A was the single-detectable type of the NS gene within the studied isolates. All the examined Iranian isolates fell into the Korean sublineage with a relatively broad sequence homology (91.6-98%) in nucleotide construction of the NS genes. The motif for PDZ ligand recognition of the group one isolates was either EDEV (N=6) or ESEV (N=1) While all viruses as group two contained a PL motif "KSEV" (N=3). The present work provides useful epidemiological data at molecular level on source and contemporary evolution of H9N2 virus population in Iran.

***Keywords:*** *Phylogenetic profiling, Influenza Virus, H9N2, NS gene*

## INTRODUCTION

Iranian poultry industry has been frequently affected by avian influenza viruses causing considerable economic losses by reducing egg production and high mortality rate (Vasfi Marandi & Bozorgmehri Fard 2002, Nili & Asasi 2002, Nili & Asasi 2003, Shoushtari *et al* 2007, Fereidouni *et al* 2010), of which H9N2 subtype avian influenza viruses have been the most prevalent (Nili & Asasi 2002, Soltanialvar *et al* 2010). The genome of avian influenza viruses composes of eight separate segments of single-stranded, negative-sense RNA coding for 10 to 11 viral proteins (Inglis *et al* 1979, Enami Sato *et al* 1994, Koparde & Singh 2011). The smallest segment (segment 8) with 890 nucleotides contains two overlapping reading frames that encode for two nonstructural proteins, NS1 and NS2. The NS1 protein consists of 124 – 237 amino acids, depending on the virus strain. NS1 is a

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multifunctional protein that plays an important role in the virus replication (Greenspan, Palese *et al* 1988, Enami, Sato *et al* 1994, Banet-Noach *et al* 2007) and in virulence by protecting the virus against cellular cytokine responses (Qiu & Krug 1994; Marión et al. 1997, Suarez & Perdue 1998, Neumann *et al* 2000, Garaigorta & Ortín 2007). The NS2 protein is known as a nuclear export protein (NEP) in transporting newly synthesized virion RNAs from the nucleus to the cytoplasm by acting as an adaptor between viral ribonucleoprotein complexes and the nuclear export system of the cell. NS2 contains a leucine-rich nuclear-export signal that could complete directional nucleocytoplasmic trafficking by helping viral ribonucleoproteins to cross the nuclear pore complex (Yasuda, Nakada et al. 1993; O'Neill, Talon et al. 1998; Banet-Noach, Panshin et al. 2007; Hale, Randall et al. 2008). In the present study, the sequence of the NS genes of the H9N2 influenza virus strains isolated during 1988-2007 in Iran was characterized and phylogenically analyzed.

## MATERIALS AND METHODS

**Isolation of Avian Influenza A H9N2 Subtype.** Avian influenza A H9N2 viruses were isolated from commercial chicken flocks in the different provinces of Iran during 1998–2007. The isolates were propagated in ten-day-old specific pathogen free embryonated chicken eggs. Haemagglutination inhibition) HI) and neuraminidase inhibition (NI) tests were used for antigenic identification of the isolates. Identification was further confirmed using RT–PCR method. The pathogenicity of the H9N2 isolates was determined by the means of intravenous pathogenicity index (IVPI) test. Allantoic fluids were harvested and used as virus stock for further studies.

**RNA Extraction and RT-PCR Assay.**Viral RNA was extracted from the HI positive allantoic fluids of the infected embryonated eggs using a high pure nucleic acid kit (Roche, Penzberg, Germany) according to the manufacturer's instruction. Reverse transcription was carried out using the Titan one- step RT-PCR kit (Roche, Penzberg, Germany) and the universal primers adapted from (Li *et al*, Li, Jiang *et al* 2006). Following a reverse transcription step at 45 °C for 45 min and an initial denaturation step at 92 °C for 2 min, PCR amplification reactions were carried out in 35 cycles of 30 s at 94 °C; 45 s at 57 °C; and 2 min at 68 ˚C for 2 min; and a final extension at 68 °C for 10 min.

**Sequence Analysis.** DNA fragments of the expected length were extracted and purified from 1% Agarose gel using High Pure PCR product purification kit (Roche, Penzberg, Germany). The purified DNA fragments were quantified and directly sequenced by MWG Biotech (Ebensburg, Germany). DNASTAR and BioEdit package version 5 were used for the assembly and translation of the nucleotide sequences into protein sequence.

**Phylogenetic Analysis.** Phylogenetic analysis was carried out by analyzing the data obtained from the sequences of the Iranian H9 isolates and some published sequences chosen from GeneBank. Consisting H9N2 isolates from Eurasian countries. Neighbor joining method with 1000 replicates of bootstrap was used to construct the phylogenetic tree. Accession number of the isolates is available (Table 1).

**Table 1.** Iranian influenza virus isolates used to study nonstructural genes.

| | Virus | Subtype | Accession number |
|---|---|---|---|
| 1 | A/chicken/Iran/28/2008 | H9N2 | HQ333523 |
| 2 | A/chicken/Iran/68/2006 | H9N2 | HQ333524 |
| 3 | A/chicken/Iran/92/2003 | H9N2 | HQ333525 |
| 4 | A/chicken/Iran/233/2001 | H9N2 | HQ333526 |
| 5 | A/chicken/Iran/320/2003 | H9N2 | HQ333527 |
| 6 | A/chicken/Iran/466/2002 | H9N2 | HQ333528 |
| 7 | A/chicken/Iran/565/2000 | H9N2 | HQ333529 |
| 8 | A/chicken/Iran/661/1998 | H9N2 | HQ333530 |
| 9 | A/chicken/Iran/772/1999 | H9N2 | HQ333531 |
| 10 | A/chicken/Iran/SH2/2007 | H9N2 | HQ333532 |

## RESULTS

**Characterization of Nucleotide and Deduced Amino Acid Sequences.**The NS1 genes of all H9N2 isolates were sequenced and phylogenetically analyzed.

The NS1 and NS2 proteins are encoded by the smallest genomic RNA segment 8, which contains 890 nucleotides (Figure 1). The nucleotide sequences of the NS segments showed 91.2% - 98.7% homology within

the H9N2 isolates (Table 2). The homologies of the NS1 and NS2 proteins within the H9N2 isolates were 89.1%-99.6% and 91.5%-99.2%, respectively.

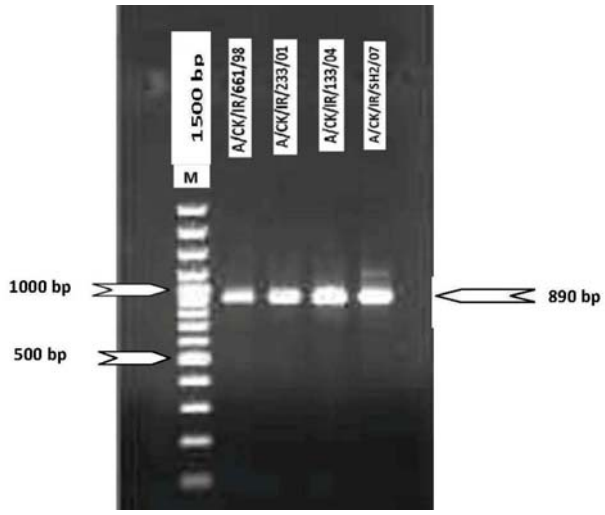


**Figure 1.** Full length amplification of the NS segments. The length of NS = 980 bp. "M" represents molecular marker =1500 bp. Numbers 1–4 represent A/Chicken/Iran/661/98 (H9N2), A/Chicken/ Iran/233/01 (H9N2), A/Chicken/Iran/133/04 (H9N2), and A/ Chicken/ Iran/SH2/07 (H9N2).

**Characterization of amino acid sequences of the NS1 and NS2 Proteins.** The NS1 proteins of the H9N2 isolate contained 230 amino acid residues (Figure 2). The amino acid substitutions were identified in 44 positions (19%) in the NS1 proteins of the H9N2 isolates, while the other 186 amino acid residues were conserved. The most percentage of amino acid substitutions (21 %) in NS1 were found in the C-terminal domain, between positions 74 and 230, and the least in the N-terminal (1-73 residue), especially between positions 1 to 50 (14%) (Figure 2). The NS2 proteins of all the H9N2 isolates were shown to have 121 amino acid residues without any insertions or deletions. The NS gene of most avian influenza , all human, swine , equine and also all Iranian H9N2 viruses isolated in this study are belonged to Allele A (Figure 3). As shown in Table 2, 89.3%-100% homology were observed between the NS genes of the Iranian H9 isolates with the viruses of allele A, while the homology between NS genes of these isolates with the viruses of allele B were 70.4%-72.% . Similar result was obtained from the analysis of the NS1 amino acid residues (data not shown). The NS1 protein binding motif from avian influenza viruses in this study has the consensus sequence ESEV, EDEV or KSEV. The NS1 protein from viruses in group one except {A/CK/IR/661/1998 (H9N2) (ESEV )} have EDEV protein binding motif but NS1 protein from all viral isolates in group two and also A/CK/Karachi/NARC-100/04 (H7N3) virus from Pakistan have KSEV protein binding motif (Figure 2).

**Phylogenetic analysis.**All viruses isolated in this study based on NS gene are divided into two distinct groups, Group one consists of viruses isolated during 1998-2003 and 2007 which were clustered with viruses A/mallard/Netherlands/14/2001 (H2N3) and A/mallard/ Sweden/98/02 (H1N6) from the Netherlands and Sweden, while group 2 including viruses isolated between 2003 and 2006 shared more similarity to viruses from UAE and Pakistan (Figure 3). It is interesting that, two viruses isolated in 2003 were grouped in different clusters (Figure 3).

## DISCUSSION

NS gene of avian influenza H9N2 virus codes for two nonstructural proteins (NS1 and NS2). The NS2 is frequently referred to as the 'nuclear export protein' (NEP) for its role in transporting newly synthesized RNP from the nucleus to the cytoplasm (O'Neill *et al.* 1998) while, NS1 effects on a vast range of cellular activities such as RNA transporting, splicing and translation. In the present study, the NS genes often H9N2 viruses isolated from commercial chicken flocks in Iran during 1998-2007 have been completely sequenced and phylogenetically analyzed. Based on nucleotide sequence homology, the NS genes of avian influenza viruses are divided into two alleles A and B (Wang, Shi et al. 2005; Lin, Lan et al. 2007) and Iranian H9N2 isolates were grouped in allele A (Figure 3). The deduced amino acid sequences homology of the NS proteins of viruses in this study was between 91.2% - 98.7% which was in the range of Allele A amino acid identity 93% - 100%. Assuming that Allele B is the old form of NS1 protein therefore

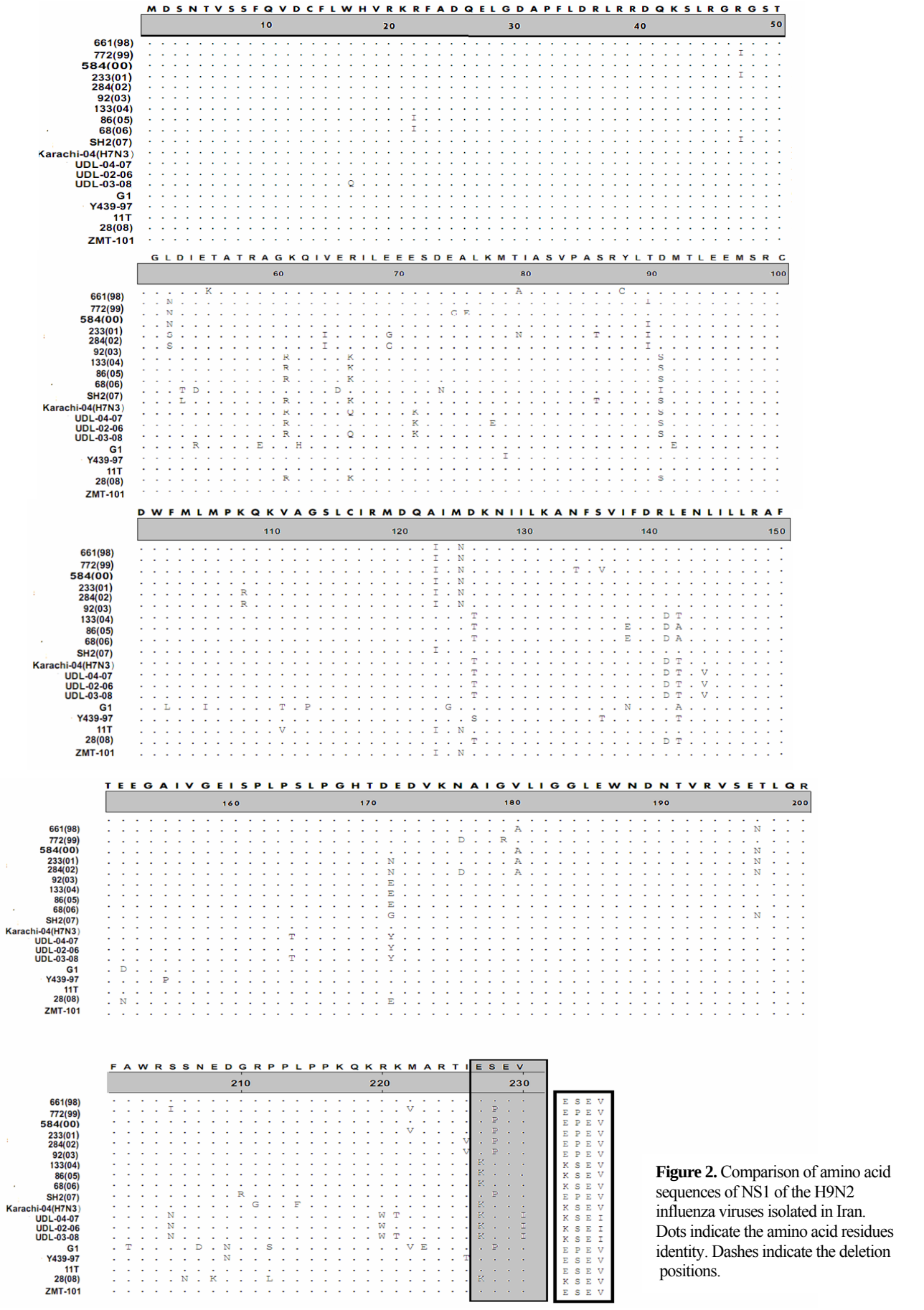


**Figure 2.** Comparison of amino acid sequences of NS1 of the H9N2 influenza viruses isolated in Iran. Dots indicate the amino acid residues identity. Dashes indicate the deletion positions.

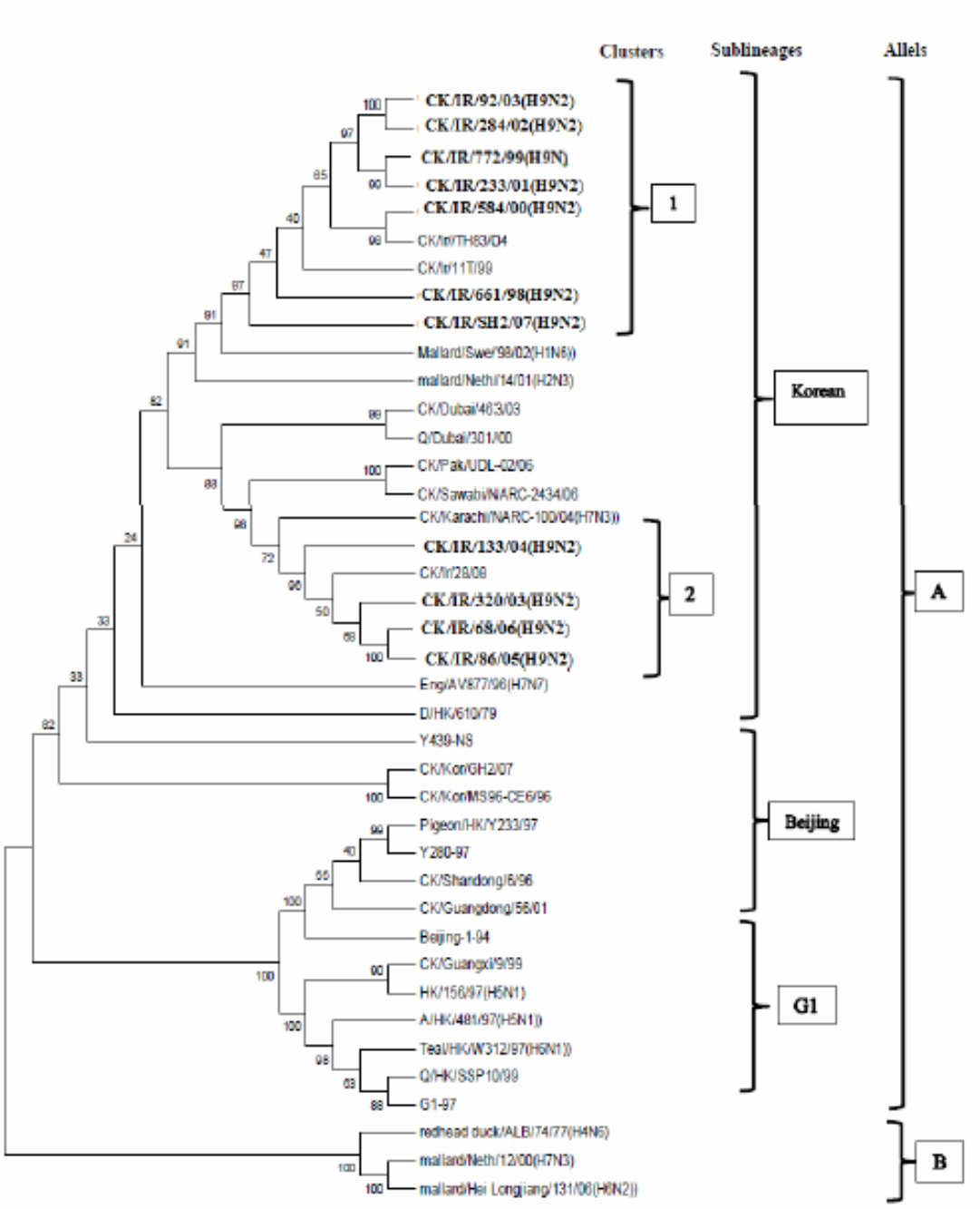


Figure 3. Phylogenetic relationships of influenza A viruses based on nucleotide sequence of the nonstructural gene that were generated by using the Neighbour Joining method. Abbreviations used for identifying isolates include CK (chicken), IR (Iran), Pak (Pakistan), DK (duck).

substitution of NS gene in Allele A H9N2 viruses seems to be for more adaptation. Phylogenetic Analysis of the NS genes showed that despite the variety of the viruses isolated in this study, they are divided into two distinct groups under the Korean sublineage (Figure 3).The close proximity of viruses from other countries to viruses in this study; show that separation of these two groups has been happening before entering into the country. The two viruses A/CK/Pakistan/UDL 04/2007 (N9N2) and A/mallard/Netherlands/14/2001 (H2N3) have been isolated in Pakistan and Netherlands in 2007 were grouped with viruses in this study but each of

**Table 2.** Comparison between Iranian isolates and representative samples of influenza A viruses belonging to different groups.

| Viruses | Homology (%) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Allele A | | | Allele B | | | Viruses closely related with | | | | | |
| | | | | | | | Iran H9N2 isolates | | | Iran H5N1 isolates | | |
| | Min | Max | Ave | Min | Max | Ave | Min | Max | Ave | Min | Max | Ave |
| Allele A | 88.5 | 100 | 94.3 | | | | | | | | | |
| Allele B | 69.3 | 72.0 | 70.6 | 93.9 | 98.1 | 96.0 | | | | | | |
| Iran H9N2 | 89.3 | 100 | 94.6 | 70.4 | 72.0 | 71.8 | 91.7 | 98.7 | 94.2 | 91.9 | 96.4 | 94.1 |
| Viruses closely related with Iran H9N2 | 89.3 | 98.7 | 94.0 | 40.3 | 72.0 | 71.15 | 91.9 | 98.7 | 95.3 | 90.7 | 95.7 | 93.2 |
| Iran H5N1 | 89.4 | 97.3 | 93.3 | 71.4 | 71.8 | 71.6 | 91.9 | 94.0 | 92.9 | 99.6 | 99.6 | 99.6 |
| Viruses closely related with Iran H5N1 | 88.6 | 96.5 | 92.5 | 71.4 | 71.8 | 71.6 | 91.9 | 94.0 | 92.0 | 99.4 | 100 | 99.7 |
| NS1 protein of IranH9N2 isolates | | | | | | | 86.5 | 97.4 | 91.9 | | | |
| NS1 protein of IranH5N1 isolates | | | | | | | | | | 100 | 100 | 100 |

them with one of these two clusters (Figure 3). The NS gene of the second group of viruses in this study was clustered with NS gene of A/CK/Karachi/NARC-100/04 (H7N3) virus and reveal that NS gene has been exchanged between these two viruses (H7N7 and H9N2) (Vasfi Marandi, Bozorgmehri Fard) (2002) .The maximum similarity was found between A/CK/IR/86/05 (H9N2) and A/CK/IR/68/06 (H9N2) virus to 100 percent, which imply that NS genes of these viruses have no changes. The least similarity was found between A/CK/IR/284/02 (H9N2) from group one and A/CK/IR/86/05 (H9N2) and A/CK/IR/68/06 (H9N2) from group two with 92.3 percent identity which shows the entrance of different viruses to the country. Since the Iranian isolates belong to two distinct groups, therefore H9N2 viruses have been entered into the country twice and each time lead to the creation of a new H9N2 subtype. The NS gene of A/CK/Karachi/NARC-100/2004 (H7N3) virus showed high similarity to group 2 of viruses in this study represented by A/CK/133/2004 (H9N2) with 97/8% homology. It indicates the common origin of these two viruses. Obviously this participation can be extended into other viruses in group 2 of this study. It is interesting that based on NS gene the A/CK/Karachi/ NARC-100/2004 (H7N3) virus from Pakistan is more similar to Iranian isolates than other H9N2 viruses from Pakistan. A distance of more than 6% was observed between two different clusters of H9N2 isolates, supporting the idea that these influenza viruses may come to Iran from different origins. The differences between the H9N2 isolates in one cluster showed to not exceed 3.5%. This may be due to a few point mutations without any reassortment. Our finding showed that the rate of amino acid substitutions in the NS1 proteins (6.91%) was more than those of the NS2 (5%), meaning that the NS2 proteins are more conserved than the NS1 (Figure 2). The NS1 proteins contain two main functional domains, RNA-binding site at N-terminus and effector domain at C-terminus. The RNA-binding site comprises the first 73 N-terminus amino acid residues. Typical of AIVs having ‘‘allele A’’ NS1 genes contain NLS1 (DRLRR) inside the RNA binding site at position 34–38 (Greenspan, Palese et al. 1988; Qian, Alonso-Caplen et al. 1994; Suarez and Perdue 1998; Neumann, Hughes et al. 2000; Garaigorta and Ortín 2007). The NLS1 [34] DRLRR[38] was also indentified in all the Iranian

isolates. The C-terminus effector domain contains a Nuclear Export Signal (NES) at positions 138–147, which is thought to be required for nuclear export of viral ribonucleoprotein complexes (Li, Yamakita et al. 1998). NES has been proven to have the hydrophobic residues, L144, L146 and L147 which were also identified in the Iranian isolates. The substitution of the NS gene between H9N2 and H5N1viruse or long-term co-circulation of these strains in the different types of poultry may lead to gene exchange which is mostly responsible for the great genetic diversity in H9N2 and H5N1 viruses (Zohari *et al* 2008, Iqbal *et al* 2009). Our findings showed that at least some of the H9N2 viruses circulating in Iran exhibit differences in the NS genes that may be a basis for subsequent gene transformation. The motif of PDZ ligand recognition in avian influenza viruses is usually EDEV or ESEV and the motif in CK/IR/661/1998 (H9N2) virus of this study (as the main representative virus entered Iran at that time) is ESEV (Tonikian *et al* 2008, Zarogoulidis, Kouliatsis *et al* 2011, Emadi Chashmi *et al* 2013). Some other Iranian isolates, such as A/CK/IR/ZMT-101/1998 (H9N2), CK/IR/11T/1998 (H9N2) have been isolated simultaneously with CK/IR/661/1998 (H9N2) virus, have the same motif (Pazani 2008). The motif of all other viruses in group one is EDEV. It seems that the motif has been changed to adapt to the new conditions in chicken cells, but the way and adaptation condition is not clear. The motif for PDZ ligand recognition of group 2 virus in this study is KSEV. Interestingly, unlike other Pakistani isolates, the motif of A/CK/Karachi/NARC-100/2004 (H7N3) virus is KSEV, further implies that the NS gene of A/CK/Karachi/NAR-100/2004 (H7N3) virus has a close relationship to group 2 viruses in this study. According to the extensive study of Jach et al 2008 existence of three motifs, EPEV, ESPE and KSEV in NS1 protein of avian influenza viruses will increase the virulence of the virus in mice. These observations, confirm the Ohenar and colleagues hypothesis about the existence of birds origin NS1 protein in human cells may facilitate the interaction of these proteins with PDZ ligand proteins which increase the virulence of the virus. So it can be concluded that the NS gene for human cells PDZ ligand recognition are in the first steps of changing in the cells of land birds (Neumann, Noda *et al* 2009; Neumann, Chen *et al* 2010; Thomas, Kranjec *et al* 2011). Although the circulation of H9N2 subtype in the mammal population (pigs and humans) increase the adaptability of H9N2 virus as a new human subtype but More importantly (over the time) by adaption of H9N2 virus to human cells further gene rearrangement opportunity are provided by common viruses such (H1N1 and H3N2) and rising the virulence. In general, we conclude that all influenza virus genes isolated in Iran in this study were originated from Southeast Asia., Where influenza viruses created new generation by reassortment. These new viruses transmitted to the Middle East by wild migratory birds or trade and gene exchange between influenza viruses were continued in the Middle East countries vigorously and created new subtypes. This phenomenon has been clearly shown in this study and the necessity of a sustained molecular epidemiological surveillance for avian influenza viruses is obvious.

## Conflict of Interest

Hereby, I declare "no conflict of interest exists" regarding submitted article.

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