Molecular characterization of non-structural gene of H9N2 subtype of avian influenza viruses isolated from broiler chickens in Iran

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Abstract:

BACKGROUND: The H9N2 subtype of avian influenza viruses (AIVs) have spread in Asia and Middle East countries and have become a serious threat to poultry industry in Iran. **OBJECTIVES:** Characterization of genes of H9N2 subtype involving in pathogenicity and diagnosis are crucial in control of avian influenza outbreaks. The Nonstructural (NS) gene and its protein products (NS1 & NS2) are important as diagnostic marker, life cycle and pathogenicity of AIVs. **METHODS:** The NS gene of five strains, isolated from 1998 to 2010, were completely sequenced and analyzed. **RESULTS:** All of the examined strains were composed of 890 nucleotides with 230 amino acids. In this regard, only two Iranian strains from GeneBank had 217 amino acids in NS1 protein. All Iranian H9N2 strains subdivided into two distinct sublineages including I and II. Comparative analysis of NS genes of Iranian strains showed that since 2003, they might have originated from Pakistan H7N3 strains; whereas from 2008 they could be originated from Pakistan H9N2 strains. **CONCLUSIONS:** Although the low pathogenic H9N2 subtype has been permanently circulating from 1998 to the present in Iran, phylogenetic analysis of NS genes revealed that sublineage II has circulated more in poultry industry of Iran. These epidemio-logically variations could be related to vaccination pressure due to massive vaccination or NS gene reassortment in rural and backyard chickens.

Introduction

Avian influenza (AI) is an important infectious disease belonging to type A influenza virus of Orthomyxoviridae family. They contain a single-stranded, negative sense, segmented RNA genome consisting of eight segments, which encode 11 known proteins. Avian influenza virus (AIV) is classified into subtypes depending upon their surface haemagglutinin (HA) and neuraminidase (NA) glycoproteins (Palese & Shaw 2007). To date, 16 HA subtypes (H1- H16) and 9 NA subtypes (N1 - N9) are recognized (Zohari et al., 2008). The AIV remains an important concern both in animal and human health (Capua and Alexander, 2008). Waterfowl is a natural host for AIVs, but they also infect and cause disease in humans and other animals such as pigs, horses and various bird species (Webster et al., 1992). Recently, some subtypes like H5, H7 and H9 were able to adapt and be established in commercial and domestic poultry and cause sever to mild disease (Alexander et al., 2007, Xu et al., 2007). The subtypes of H5 and H7 can be assumed as highly pathogenic avian influenza (HPAI), but H9 subtypes are recognized only as low pathogenic avian influenza (LPAI) and all of these subtypes are highlighted for their ability in inducing

zoonotic infection (Guo et al., 1999, Butt et al., 2005).

The AIVs of H9N2 subtype have been isolated from poultry outbreaks in several countries including Germany, Iran, Pakistan, Italy, Ireland, Saudi Arabia, China, Hong Kong, South Africa, and the United States during the last decade (Naeem et al., 1999, Lee et al., 2000). In 1998 for the first time, H9N2 subtype of AIV was isolated in Iran by Vasfi Marandi and Bozorgmehrifard (Marandi, M., Bozorgmehrifard, M.H., 1999). This outbreak caused great economic losses in Iran poultry industry. The mortality rates reported in some broiler farms in Iran were up to 65% (Nili & Asasi, 2002).

The segment 8 of AIV gene is about 890 nucleotides in length and codes 2 non-structural protein 1 and 2 (NS1and NS2). The NS1protein is not present in mature virions but is visible in infected cells, whereas the NS2 protein presents in very low rate in the viral particle (Salvatore et al., 2002). Depending on virus strains, NS1 protein consists of 124-237 amino acid residues. The mRNA of NS1 directly encodes NS1 protein but for production of NS2 protein, splicing of mRNA of NS1 leads to the formation of the NS2 mRNA which encodes 120 amino acids residues (Matrosovich et al., 2004, Lye et al., 2006). The NS genes of influenza viruses are divided into two alleles groups, designated as alleles A and B. Allele A comprise influenza viruses from human, equine, swine and bird species, whereas allele B comprises one equine and many bird influenza isolates (Treanor et al., 1989, Ludwig et al., 1991). The level of homology within allele A and B is 93-100%, and those between two alleles are about 62% (Treanor et al., 1989).

The viral NS1 protein is widely considered as the common factor by which all influenza A viruses antagonize host immune responses. Indeed, mutant influenza A viruses unable to express NS1 protein alone, display high pathogenicity in mice lacking antiviral mediators or the dsRNA- activated protein kinase (PKR). Thus, the available data strongly indicate that the major function of NS1 in current *In vivo* models is to antagonize IFN-α/β mediated antiviral response (Hale et al., 2008a). The NS1 protein is expressed like two distinct functional domain including RNA- binding and effector domains. The RNA-binding domain is localized in the N-terminal region (first 73 amino acid residues) and effector domain is situated in C-terminal region (from 74 to the end). The effector domain is programmed for interaction with a long series of viral and cellular factors (Hale et al., 2008b, Mario et al., 1997). The NS protein is a very useful target internal protein that has been involved in differentiation of infected vaccinated animal from those of uninfected vaccinated animals (Dundon & Capua, 2009). Therefore, detection of important molecular determinants on NS gene of H9N2 protein could be helpful in development of diagnostic tests. So, the aim of this study is molecular identification and characterization of NS genes Iranian H9N2 subtypes strains of AIV isolated from broiler chicken flocks between 1998-2010.

Materials and Methods

Virus strains: Four of the five AIVs used in this study were isolated in avian virology laboratory of the Faculty of Veterinary Medicine in Tehran University from 2006 until 2009 during AI outbreaks in broiler chicken farms in Tehran province. Besides, the ZMT-101 strain as the Iranian reference strain was isolated at the first H9N2 outbreak in 1998. Tissue samples were collected according to the standard method from suspected broiler chickens. The lung and intestine were sampled and stored at-70°C until virus isolation. Samples were prepared in a 2x phosphate buffer solution (PBS, pH 7.4) containing antibiotics (10.000 IU/mL penicillin, 1mg/mL streptomycin sulfate) and antifungal (20 IU/mL Nystatin) (SIGMA, St. Louis, MO, USA). Then, AIV homogenized tissue samples were inoculated in 10 day-old embryonated chicken eggs (Kendal et al., 1982, Peiris et al., 1999). The subtypes of AIVs were detected by specific H9N2 subtype antiserum using hemagglutination inhibition (HI) test (Istituto Zooprofilattico Sperimentaledellen Venezie, padova, Italy). Allantoeic fluids containing AIVs with Log 2 HA titer of 7 were used for RNA extraction. The H9N2 strains used in this study is listed in Table 1.

RNA extraction and RT-PCR: The viral RNA was directly extracted from allantoic fluid possessing H9N2 subtype of AIV with the RNX-TM- PLUS kit (Cina Gene Com. Iran). Reverse transcription (RT) was carried out with uni-12 universal primer with sequence of 5'AGAAAAAGCAGG3'. Amplific-

ation of the NS genes was performed by PCR, using specific primers. All the primers used in this study are available in the avian virology laboratory of the Faculty of Veterinary Medicine in Tehran University.

The reaction mixture with total volume of 50 µL contained 5 µL of cDNA, 15 p moles of forward and reverse primers (4 µL) and 25 µL of PCR master mix (Cina Gen, Com). The amplification protocol was: one step of denaturation at 94°C for 4 min, 30 cycles of 94°C/20 Sec, 50°C /30 Sec, and 72°C/120 Sec, and final extension at 72°C for 7 min.

The PCR products were separated by electrophoresis using 1% agarose gel. The PCR products were purified with the QIA quick Gel Extraction Kit (Qiagen, Valencia, CA, USA) and sent for sequencing (SinaClon company). The nucleotide sequences of five NS genes obtained in this studywere submitted in GeneBank database and are available under accession numbers of JQ364984, JQ364985, JQ364986, JQ364987 and JQ364988 (Table 1).

Further, other sequences of NS genes of influenza viruses were obtained from GeneBank and used in characterization and phylogenetic analysis, based on nucleotide and amino acids sequences of the NS genes. The Phylogenetic tress were drawn regarding complete open reading frame (ORF) of all strains with 1000 boots trapping replications in Molecular Evolutionary Genetics Analysis (MEGA, version 4. 1 beta) software (Tamura et al., 2007).

Results

NS gene homology: The NS segments of the 5 isolates of H9N2 strains were completely sequenced. The results showed that the lengths of the RNA region coding NS1 and NS2 proteins in all isolates were 835. The NS1 gene nucleotide sequence homology between them was 95-100 % with an average of 97 % and those compared for deduced amino acids homology were 89-100% with an average of 94%. The nucleotide and amino acid sequence homology of the NS1 genes of these isolates and other Iranian available strains in GeneBank were respectively 89-100 % with an average of 94% and 82-100% with an average of 91.7 %. (Table 2).

Among the NS genes of Iranian H9N2 strains there were only 2 strains named A/chicken/Iran/TH85/2006, A/chicken/Iran/TH186/2007, which contained 217 amino acid residues with 13 amino acids in C-terminal truncation.

A total of 150 positions from 230 deduced amino acids (65.2 %) were conserved in NS1 protein of all Iranian strains. However only 30 positions with 87% conservation were found in five strains. The RNA-binding domain of NS1 protein was composed of 73 amino acids, of which 49 positions (67%) are conserved in all Iranian strains. The effector domain consisted of 157 amino acids. In which, positions 74 to 230 contained 101 positions (64.5%) that are conserved, but in five strains, amino acid conservation in RNA binding domain and effector domains were 89% and 86% respectively (Table 3).

The NLS1 sequence contained the stretch of basic amino acids sequence 34-DRLRR-38 which was located in RNA- binding domain. It was conserved among the 5 H9N2 subtype strains analyzed in this study.

The amino acid of positions 219 and 220 in A/Chicken/Iran/ZMT-101/1998 and A/Chicken/EBGV-86/2007 strains were respectively Lys and Arg, but in A/Chicken/EBGV-87/2008, A/Chicken/EBGV-88/2010 and A/Chicken/EBGV-89/2010 strains were Lys and Trp respectively. The amino acid positions of 89, 93, 167 and 141 are conserved in five strains, but with the exception of A/Chicken/EBGV-87/2008 strain, all strains possessed proline in position 164. In A/Chicken/EBGV-87/2008 strain proline is substituted with His. A/Chicken/Iran/ZMT-101/1998 strain and all Iranian strains isolated before 2003 possessed Glu in position 142, but all Iranian strains isolated after 2003 (A/Chicken/Iran/EBGV-86/2007, A/Chicken/Iran/EBGV- 87/2008, A/Chicken/Iran/EBGV-88/2010 and A/Chicken/Iran/EBGV-89/2010) had Asp in position 142. All Iranian strains contained Phe, Met and Leu in the positions of 103, 106 and 144 respectively. However, regarding the amino acid residues at positions of 184-188, it was slightly different in A/Chicken/Iran/EBGV-87/2008 strain in which the residues of GLVWN were substituted with GLEWN (Table 4).

The PDZ Ligand or PL motives of ESEV, KSEV, KSEI, EPEV and LPPK were found among NS genes of all Iranian strains available in GeneBank. The PL motives in A/ Chicken/ Iran/ ZMT-101/ 1998, A/ Chicken/ Iran/ EBGV-86/ 2007, A/ Chicken/ Iran/ EBGV-87/2008 and A/ Chicken/ Iran/ EBGV-89/

Table 1. Iranian strains characterized in this study.

No	Virus Strain	Accession Number
1	A/Chicken/Iran/ZMT- 101/1998(H9N2)	JQ364985
2	A/Chicken/Iran/EBGV-86/2007(H9N2)	JQ364986
3	A/Chicken/Iran/EBGV-87/2008(H9N2)	JQ364987
4	A/Chicken/Iran/EBGV-88/2010(H9N2)	JQ364984
5	A/Chicken/Iran/EBGV-89/2010(H9N2)	JQ364988

2010 strains used in this study were respectively ESEV, KSEV and KSEI (Table 5).

Phylogenetic analysis: The phylogenetic tree of NS genes of five H9N2 strains isolated during 1998-2010 from different farms (presented in this study) in Tehran province in Iran are shown into two small groups (Figure 1). One group included A/Chicken/ Iran/ZMT-101/1998 and A/Chicken/Iran/EBGV-86/2007 strains, whereas the other group possessed A/Chicken/Iran/EBGV-87/2008, A/ Chicken/ Iran/ EBGV-88/ 2010, and A/ Chicken/ Iran/ EBGV-89/ 2010 strains (Figure 1). Also, various sequences of NS gene of Iranian H9N2 strains and some other subtype related sequences were obtained from the Gene Bank and used for phylogenetic analysis (Figure 2,3). The nucleotide sequences of NS1 coding region showed that all Iranian strains could be divided into two sub-lineages of I and II from 1998 to 2010. In sub-lineage I of Iranian strains includ A/ Chicken/ Iran/ ZMT-101/ 1998, A/ Chicken/ Iran/ 11T/ 1999, A/Chicken/ Iran/ 772/ 1999 and some other H9N2 strains; there was 93-100% homology with an average of 93.3 % in NS1 gene and 86-100% homology with an average of 93% in deduced amino acid sequence of NS1 protein (Table 2). However, in sub-lineage II strains including A/ Chicken/ Iran/ EBGV-86/ 2007, A/ Chicken/ Iran/ EBGV-87/ 2008, A/ Chicken/ Iran/ EBGV-88/ 2010, A/ Chicken/ Iran/ EBGV-89/ 2010, A/ Chicken/ Iran/ TH186/ 2007 and some other strains isolated from 2003 there was 92-100% homology with an average of 95.7% for NS1 gene nucleotides sequence, and 88-100% homology with an average of 94.6% in NS1 protein (Table 2). Sub-lineage I contained all AIVs isolated from 1998 to 2003, whereas in sub-lineage II there were two distinct clades, one clade only possessing A/ Chicken/ Iran/ EBGV-87/ 2008, A/ Chicken/ Iran/ EBGV-88/ 2010 and A/ Chicken/ Iran/ EBGV-89/ 2010 (Figure 2).

According to alleles A and B among the NS genes nucleotide sequence homology of H9N2 strains from different parts of the world, as previously described by Treanor et al., (1989), all Iranian stains including 5 study strains were located in allele A (Figure 3).

Phylogenetic tree based on full length of NS drew between representative H9N2 strains from Asia including China, Hong Kong, Korea, Pakistan, India strains and Middle East countries such as A/ Quail/ Dubai/302/2000 and A/Chicken/Israel/292/ 2008 and Iranian strains including all five strains and A/ Chicken/ Iran/ 28/2008(H9N2), A/ Chicken/ Iran/ 320/ 2003(H9N2), A/ Chicken/ Iran/772/ 1999 (H9N2), A/ Chicken/ Iran/ 11T/ 1999 (H9N2), A/ Chicken/ Iran/ 53-3/2008(H5N1), A/ Cygnus Cygnus/ Iran/ 754/ 2006 (H5N1), showed that these strains also established Eurasian H9N2 lineages: namely the G1-lineage, the Y280-lineage and the Korean-lineage represented by prototype viruses of A/ Quail/ Hong Kong/ G1/97, A/ Duck/ Hong Kong/ Y280/97, A/ duck/ Hong Kong/ Y439/1997 and A/ Chicken/ Korea/ 38349p96323/96 respectively (Figure 3).

Discussion

Since the occurrence of the first AI outbreak due to H9N2 subtype in Iran in 1998, frequent incidences of H9N2 outbreaks have, in recent years, caused high mortality in broiler chicken farms in Iran and some other Asian countries, resulting in considerable economic losses (Marandi and Bozorgmehri Fard, 1999, Haghighat-Jahromi et al., 2008). In spite of the HA gene characterization of the H9N2 viruses isolated in Iran, only a few molecular analyses of the NS genes have been reported.

The previously available NS gene of Iranian H9N2 and H5N1 subtypes sequences in Gene Bank and 5 strains isolated from 1998 until 2010 were investigated regarding their nucleotide and amino acid sequences. Phylogenetic analysis based on open reading frame (ORF) of NS1 gene of all Iranian H9N2 strains showed the existence of two main sub-lineages from 1998 to 2010 including sub-lineage I and II. The ZMT-101 strain belongs to sub-lineage I, and other H9N2 strains of this study fell in sub-lineage II (Figure 2). Phylogenetic tree showed that Iranian H9N2 strains located in sub-lineage II were

Table 2. Nucleotide and amino acid sequences homology of 23 Iranian strains (all Gene Bank strains and five study strains).

Homology	NS gene		NS1 gene		NS1 protein	
	range %	average %	range %	average %	range %	average %
Five strains	94-100	95.5	95-100	97	89-100	94
All Iranian strains	88-100	94.1	89-100	94	82-100	91.7
Sub-lineage I of Iranian strains			93-100	93.3	86-100	93
Sub-lineage II of Iranian strains			92-100	95.7	88-100	94.6

Table 3. Amino acids (aa) conservation and its percentage (%) in NS1 protein, RNA-binding and effector domain of 23 Iranian strains.

Conservation	NS1 protein		RNA-binding domain		Effector domain	
	aa	%	aa	%	aa	%
Five strains	200	87	65	89	136	86
All Iranian strains	150	65.2	49	67	101	64.5

Table 4. This table shows the amino acid (aa) position in NS1 protein of Iranian strains.

Amino acid position	142	164	184-188	219	220
A/Chicken/Iran/ZMT-101/1998	E	P	GLEWN	K	R
A/Chicken/Iran/EBGV-86/2007	D	P	GLEWN	K	R
A/Chicken/Iran/EBGV-87/2008	D	H	GLVWN	K	W
A/Chicken/Iran/EBGV-88/2010	D	P	GLEWN	K	W
A/Chicken/Iran/EBGV-89/2010	D	P	GLEWN	K	W

isolated from 2003; whereas all strains isolated until 2004 with one exception of A/chicken/Iran/SH2/ 2007, were located in sub-lineage I.

When the NS gene sequences of 5 isolates of H9N2 subtype were used in phylogenetic analysis with NS genes of reference subtypes of influenza viruses in NCBI blast, there was a close homology between NS genes of H9N2 and H5N1 (Figure 3).

The results of this study showed that NS genes of all Iranian isolates fall into allele A, with two separate clusters; However, the NS genes of the Iranian H5N1 isolates formed a single cluster with those of the H5N1 viruses isolated from Afghanistan and some of the H9N2 isolates from Pakistan. This phenomenon has been emphasized with Amir et al., in 2000, Banet-Noach, et al., in 2007 and Iqbal et al., in 2009.

All Iranian H9N2 strains in phylogenetic tree were located in two sub-lineages. The sub-lineage I of Iranian strains possessed A/CK/Iran/11T/1999 (H9N2) and Saudi Arabia/CP7/1998 (H9N2) strain (Figure 3). Moreover, the influenza viruses found in Northern Europe such as A/teal/Germany/Wv632/ 2005 (H5N1) and A/Mallard/Netherlands/2005 (H5N2) strains were located in the same lineage. However, the sublineage II of Iranian strains was located in a branch that consists of United Emirate and Pakistan strains. By careful investigation, sub-linage II of Iranian strains were divided into two distinct groups, both of which are closely related to Pakistan strains.

It seems that the first isolated H9N2 strain like A/ Chicken/ ZMT-101/1998 (H9N2) was the precursor for A/ teal/ Germany/ Wv632/ 2005 (H5N1) and A/Mallard/Netherlands/2005 (H5N2) strains isolated in the Netherlands and Germany in 2005. It is not strange because birds naturally migrate from southern to northern countries and genetic reassortment may be occur in their seasonal residence, because NS gene identity of H5N1 and H5N2 strains of the Netherlands and Germany with H9N2 subtype of Iranian strains such as A/Chicken/ ZMT-101/1998 (H9N2) which belong to sub-linage I, is about 98-99%.

The NS1 gene of A/Chicken/Iran/EBGV-86/2007 (H9N2) strain is very similar to A/ CK/Karachi/ NARC-100/ 2004 (H7N3) and A/ Chicken/ Iran/ EBGV-87/ 2008 (H9N2), A/ chicken/ Iran/ EBGV-88/2008 (H9N2) and A/ chicken/ Iran/ EBGV-

Table 5. this table shows PL motives of 9 Iranian and 6 Pakistani H9N2 subtype strains.

Strains name	PL motif 227-230
A/Chicken/Iran/ZMT-101/1998	ESEV
A/Chicken/Iran/EBGV-86/2007	KSEV
A/Chicken/Iran/EBGV-872008	KSEI
A/Chicken/Iran/EBGV-88/2010	KSEI
A/Chicken/Iran/EBGV-89/2010	KSEI
A/Chicken/Iran/772/1999	EPEV
A/Chicken/ Iran/565/2000	EPEV
A/Chicken/Iran/TH85/2006	LPPK
A/Chicken/Iran/TH186/2007	LPPK
A/Chicken/Pakistan/UDL-02/06	KSEI
A/Chicken/Pakistan/UDL-01/07	KSEI
A/Chicken/Pakistan/UDL-04/07	KSEI
A/Chicken/Pakistan/UDL-01/08	KSEI
A/Chicken/Pakistan/UDL-02/08	KSEI
A/Chicken/Pakistan/UDL-03/08	KSEI

Table 6. The percentage (%) of heterogeneity of 8 Iranian strains with three H9N2 subtypes strains isolated from Duck, Chicken and Quail in 1997.

Iranian strains name	A/DK/HK/Y439/1997	A/CK/HK/G9/1997	A/Quail/HK/G1/1997
A/Chicken/Iran/ZMT-101/1998	5.2%	9.9%	8.3%
A/Chicken/Iran/EBGV-86/2007	8.5%	13.5%	12.2%
A/Chicken/Iran/EBGV-872008	7.9%	12.7%	11.1%
A/Chicken/Iran/EBGV-88/2010	7.1%	12.8%	10.9%
A/Chicken/Iran/EBGV-89/2010	8.3%	13.1%	11.4%
A/Chicken/Iran/661/1998	5.5%	10.3%	8.7%
A/Chicken/Iran/TH186/2007	9.8%	15.2%	14.2%
A/Chicken/Iran/TH82/2003	9.5%	12.7%	12.2%

89/2009 (H9N2) strains are quite similar to A/ CK/ Pakistan/ UDL-04/ 2007 (H9N2) and A/ CK/ Pakistan/ UDL-02/ 2006 (H9N2) with 96% and 95.1-98.2% respectively, based on deduced amino acid sequences (Table 7). According to phylogenetic tree of A/Chicken/EBGV-86/2007 and A/chicken/Iran/320/2003 with A/ CK/ Karachi/ NARC-100/ 2004 (H7N3), it seems that NS gene of A/ chicken/ Iran/ 320/ 2003 (H9N2) and A/ chicken/ Iran/ EBGV-86/ 2007 (H9N2) have been generated from the same gene pool and genetic reassortment may occur between H9N2 and H7N3 strains. It is proposed that the H7N3 strains could have donated their NS genes to H9N2 strains as reported by Guan et al., in 1999 and Iqbal et al., in 2008. Understanding of the geographic location of this reassortment is very crucial because Iran Veterinary Organization is already doing H5, H7 and H9 surveillance. It is important to note that there is only one report of H7 subtype isolation in Iran named A/ mallard/ Iran/ V31/04 (H7). However, AIV subtype of H7 and H9 are currently endemic in Pakistan due to regular and extensive use of vaccines (Naeem et al., 1999). Therefore, more possible genetic reassortment may occur in Pakistan and a novel genotype of H9N2 introduced to Iran by migratory birds. Nevertheless, after complete sequence analysis of all 8 genes this hypothesis could be possible . This phenomenon may be true about A/ Chicken/ Iran/ EBGV-87/ 2008 (H9N2), A/ chicken/ Iran/ EBGV-88/ 2008 (H9N2) and A/ chicken/ Iran/ EBGV-89/ 2009 (H9N2) strains too. These strains are closely related to A/ Chicken/ Pakistan/ UDL-04/ 2007 (H9N2) and A/ Chicken/ Pakistan/ UDL-02/ 2006 (H9N2) strains from Pakistan. Therefore, it is not surprising to say these Iranian strains are originated from Pakistan strains. Banet-Noach, et al., in 2007 described NS genes of the Israeli H9N2 isolates originated from a single precursor and heterogenity between Israeli H9N2 strains did not exceed more than 3%, but our results were different and in some strains such heterogenity is up to 12% (Table 2). Overall, at least three distinct lineages of NS genes of the H9N2 subtype of AIV are circulating in the chicken population in Iran and 2 of their lineages have a relationship with Pakistan strains.

All Iranian strains have the same length having 230 amino acids residues, with the exception of A/chicken/Iran/TH85/2006(H9N2) and A/chicken/Iran/TH186/2007 (H9N2) strains. These later viruses are composed of 217 amino acids residues. Similar truncations have been reported previously in H7 and H9 subtypes of AIVs isolated from poultry (Dundon et al., 2006) and are more similar to A/chicken/ Iran/ EBGV-86/ 2007 (H9N2) and with 94% homology with A/ chicken/ Karachi/ NARC-100/ 2004 (H7N3). Among the H7N3 viruses with the 217 amino acid residues, all strains except one were isolated in Pakistan and Afghanistan between 1995 and 2005 (Iqbal et al., 2008, Dundon and Capua, 2009) but their homology with A/chicken/Iran/ TH85/2006 (H9N2) and A/chicken/Iran/TH186/ 2007 (H9N2) is less than

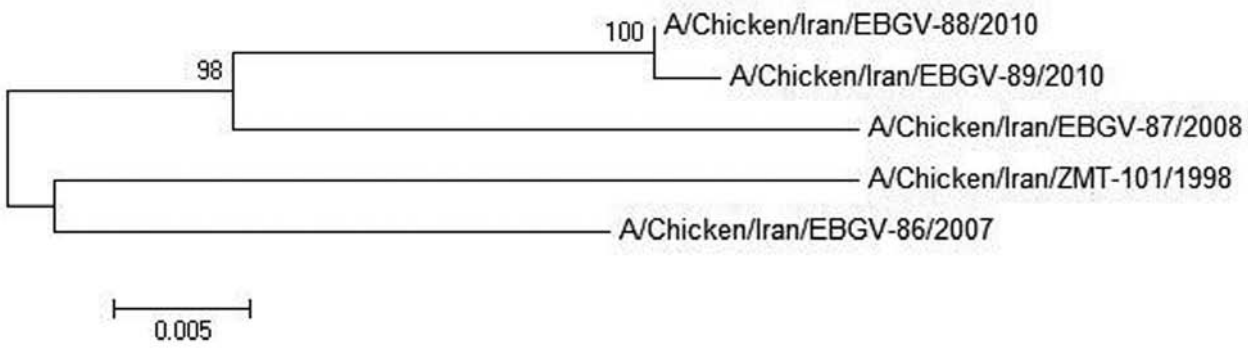

Figure 1. This figure indicates two small groups among the five H9N2 strains used in this study.

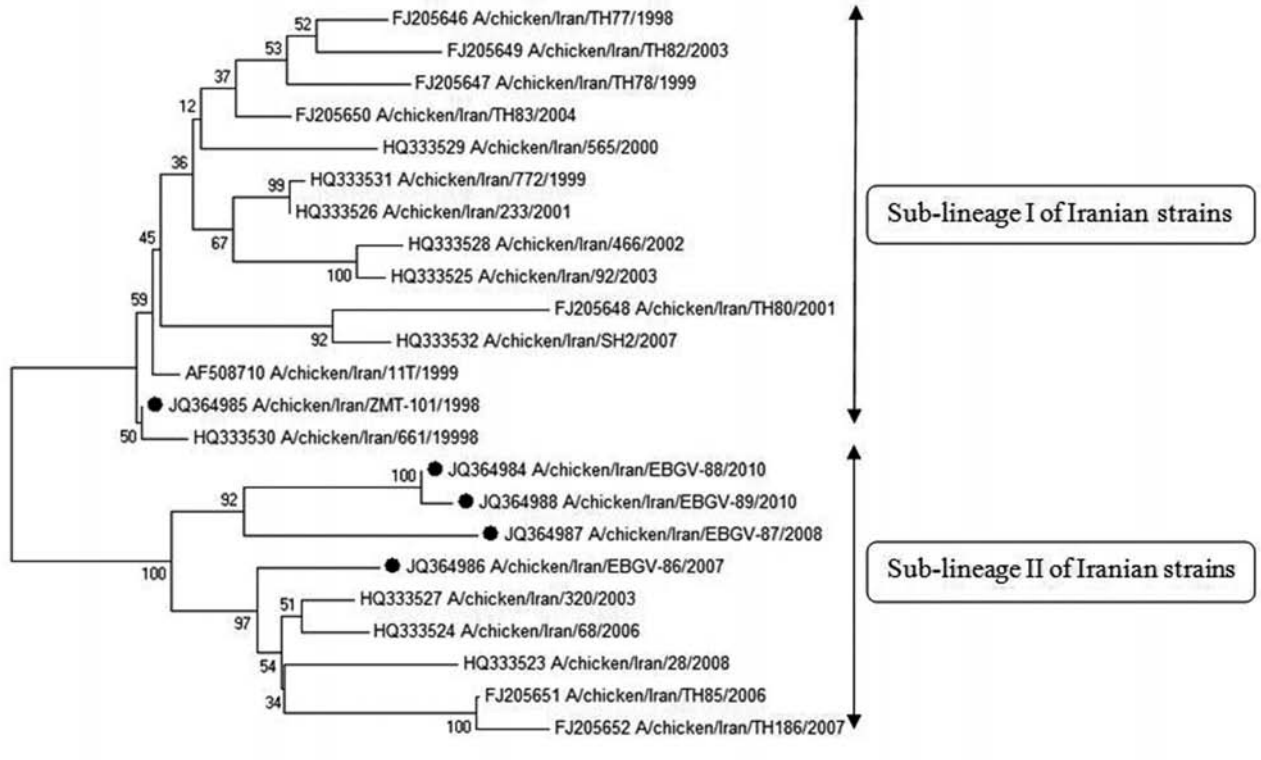

Figure 2. All Iranian H9N2 subtypes of AIVs strains phylogeny tree based on NS1 gene. The five strains used in this study shown by black circle (●). Two sub-lineages exist among all Iranian strains.

90%. All Iranian and Pakistan H9N2 subtype strains that were composed of 230 amino acids residues possessed Gln in position 218. One of the Gln nucleotides frames is CAG, on the other hand, TAG is one of the termination codon nucleotides frames. Therefore, it is possible that these strains originated from other Iranian strains with point mutation in position 218 amino acids residues. This hypothesis has been emphasized by Dundon and Capua (2009).

All NS1 proteins of H9N2 Iranian strains had amino acid residues of 80-TIASV-84, without any insertion or deletion. However, the biological

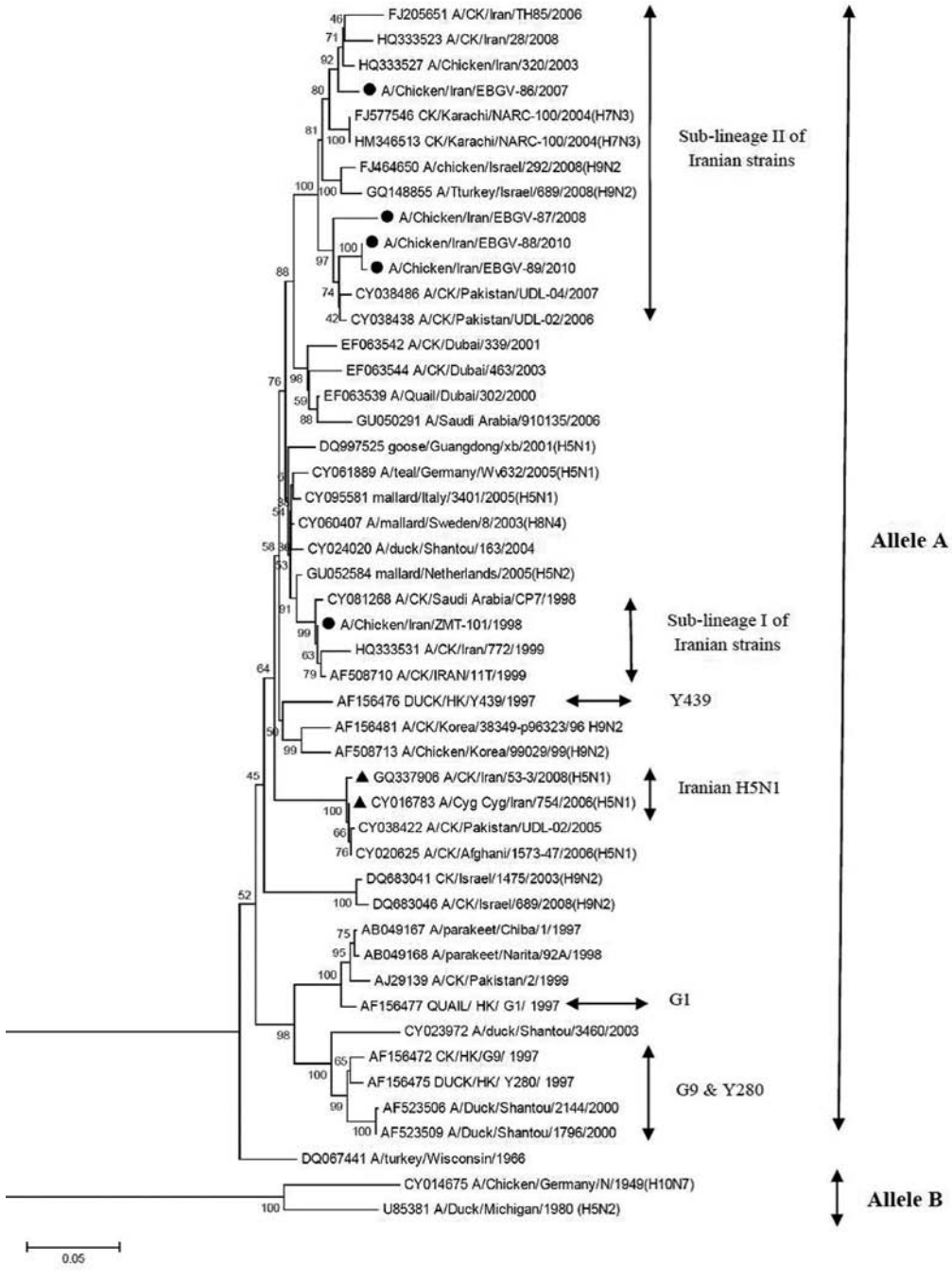

Figure 3. Phylogenetic tree of NS genes of Iranian H9N2 viruses. Black circle (●) indicates viruses characterized in this study. Black triangles (▲) are Iranian H5N1 viruses.

significance of the 5 amino acid deletion is not well understood, and the effects of such deletion on pathogenesis have become clearer. It has been reported that viruses containing NS1 with deleted 80-TIASV-84 residues show increased virulence in both mouse and poultry infections (Long et al., 2008), but this deletion in NS gene did not enhance Pakistan H9N2 strains in IVPI test (Iqbal et al., 2008).

It is not possible to clearly find Iranian precursor of H9N2 subtype strain. However, it seems a A/ duck/ Hong Kong/Y439/1997 strain might be closer to Iranian H9N2 isolates than other viruses with heterogeneity between 5.2-9.8% with Iranian strains (Table 6). If this hypothesis is correct , the proposed precursor strain may be introduced to Iran poultry industry by means of migratory birds or importation

of birds like ostrich or quail.

About 150 positions of 230 (65.2 %) deduced amino acids were conserved in NS1 protein of H9N2 subtype strains (Table 3), but this conservation in Israeli strains was about 81.7%. The divergence between the Israeli subgroups may be attributed to a few point mutations without genetic shifts (Banet-Noach, et al., 2007) but it seems that the Iranian strains overcame more divergence, emphasizing genetic shifts.

The PDZ domains are protein-protein recognition modules within a multitude of proteins that organize diverse cell-signaling assemblies. They specifically recognize and bind to short C-terminal peptide motifs of 4-5 amino acids designated as PDZ ligand (PL). The PL of avian NS1 proteins consists of residues 227-230, with the amino acids sequence of ESEV or EPEV motives. The avian PL sequence was not observed in the NS1 proteins of non-avian viruses, and for a large number of human NS1 proteins any potential PL was masked by 7 amino acid C-terminal extension (Hale et al., 2008b,c). The role of avian PL sequences on human influenza viruses virulence was recently reported (Jackson et al., 2008). Avian NS1 proteins and that of the 1918 virus are able to bind to up to 30 human PDZ domain containing proteins; however, it is not possible for human NS1 proteins (Obenauer et al., 2006). Iqbal et al., showed that 6 Pakistani H9N2 strains with PL motif were KSEI (2009) (Table 6). Besides, Iranian strains of A/ Chicken/ Iran/ EBGV-87/2008 (H9N2), A/ chicken/ Iran/ EBGV-88/2008 (H9N2) and A/ chicken/ Iran/ EBGV-89/2009 (H9N2) PL motif are also KSEI. These Iranian and Pakistani strains are in maximum homology with each other, but the origin of KSEI amino acids sequence as a PL motif is uncommon (Iqbal et al., 2009). The isoleucine residue in C-terminal of NS1 protein was rare: out of 1196 PL motif sequences only 48 sequences from avian-origin viruses and one from a swine-origin virus contained isolousine at the C-terminus. In addition, K at the position - 4 in the PL motif was also rare, with the H1N1 1918 pandemic virus (Iqbal et al., 2009). The H9N2 subtype of AIV strains from 1998 til now have been circulating in poultry industry of Iran. The PL motifs of ESEV, KSEI, KSEV, EPEV and LPPK were found in all Iranian H9N2 strains analyzed (Table 6). The variation of PL motifs in Iranian strains is more than Israel and Pakistan H9N2 strains. Such variation revealed that insertion of ESEV, EPEV, or KSEV, into an avirulent AIV strain in mouse experimental model enhanced their pathogenicity, but it is not clear whether it will enhance pathogenicity in avian host or not (Iqbal et al., 2009, Jackson et.al, 2008)

The NLS1 is nuclear signal sequence that has been localized inside the NS1 protein (Greenspan et al., 1988). The residues of 34-DRLRR-38 is conserved in NLS1 of some Iranian H9N2 subtypes of AIV strains and all Israel (Banet-Noach, et al., 2007) and Pakistan and other Middle Eastern countries strains, but in some Iranian strains isolated from 1999 to 2003, the NLS1 motif is DMLRR (A/ chicken/ Iran/ TH85/ 2006, A/ chicken/ Iran/ TH186/2007) VRRRR (A/ chicken/ Iran/ TH77/1998, A/ chicken/ Iran/ TH82/ 2003) VRLRR (A/ chicken/ Iran/ TH78/ 1999, A/ chicken/ Iran/ TH80/2001). This motif is crucial for virus replication cycle and this kind of variation must be further investigated.

The PI3K is a heterodimeric lipid kinase consisting of an 85 kDa regulatory subunit (p85) and a 110 kDa catalytic subunit (p110). After activation of P13K intracellular second messenger PIP3 is generated, which causes the specific membrane-recruitment of a diverse range of signaling proteins (Hawkins et al., 2006). The C-terminal effector domain of NS1 binds specifically and directly to the p85b regulatory isoform of PI3K (Hale et al., 2006, 2008c). To date, residues of NS1 implicated in p85b-binding include Tyr, Met, Leu, Glu, Pro and Pro in the positions of 89, 93, 141, 142, 164, and 167, which are all adjacent to one another in the NS1 monomer (Hale et.al, 2008a). All Iranian strains belonging to sub-lineage II had glutamate at position 142 that was replaced by aspartate. In position 164, only A/ Chicken/ Iran/ EBGV-87/2008 strain had histidin and all other positions were conserved in Iranian strains. These variations need to investigate the pathogenicity H9N2 strains.

CPSF30 (30 kDa subunit of cleavage and polyadenylation specificity factor) is a very important factor used by influenza virus for inhibition of RNA- polymerase II. The NS1-CPSF30 complex is thought to prevent CPSF30 from binding cellular pre-mRNAs, thereby inhibiting normal cleavage and polyadenylation of the 3' end of host-cell mRNAs, but

viral mRNAs are not affected by CPSF30 inhibition (Nemeroff et al., 1998). NS1 binding to CPSF30 appears to require Phe-103/Met-106/Leu-144 and residues 184-188. Among Iranian H9N2 strains, only A/Chicken/Iran/BGV-87/2008 possess-ed the different motif of 184- GLVWN-188 sequence. However, all other Iranian strains had 184-GLEWN-188 sequence (Table 4). It is a very important position for virulence, but its effect on virulence of Iranian H9N2 strains remains to be investigated.

Low-pathogenic H9N2 subtype of AIVs, were permanently circulating from 1998 to date in poultry industry of Iran. The NS genes of Iranian H9N2 strains isolated from1998 to 2003 are seemingly originated from A/Duck/Hong Kong /Y439/1997 (H9N2) strain, whereas those from 2003 to 2010 originated from Pakistan strains. In 2006, highly pathogenic H5N1 subtype of AIVwas introduced to Iran by migratory birds and became endemic in rural and backyard chicken of Iran. It is clearly demonstrated that genetic reassortment in AIVs is usual in wild and domestic birds (Macken et al., 2006), but the species in which genetic reassortment most readily occurs is not known for certain (Iqbal et al., 2009).

In conclusion, investigation of NS gene phylogenetic tree of Iranian H9N2 strains revealed that sub-lineage II strains are already circulating in chicken flocks. This epidemiological event could be related to vaccination pressure, because Iranian avian influenza vaccine strains are members of sub-lineage I. Moreover, the possibility of reassortment in internal genes should be considered. The genetic reassortment between H9N2, H7N3 and H5N1 subtypes of AIVs could emerge as a new genotype for subtype, as described by Iqbal et al., (2009). They demonstrated the new genotype of H9N2 strains in Pakistan that originated from Pakistan H9N2, H7N3 and H5N1 strains and showed a possible donation of H7N3 and H5N1 related NS gene to H9N2 AIV. This suggests that the surveillance of both H9 and H5 subtypes of AIVs among industrial poultry and rural or backyard chickens of Iran is very crucial in order to prevent emergence of a new genotype of H9N2 or H5N1 of AIVs. The huge spread of H9N2 to the majority of countries in the Middle East is an ongoing problem for the poultry industry. There is possibility of regeneration of a new H9N2 strain with high pathogenicity by means of reassortment. This occurrence could be succeeded by the emergence of a novel panzootic strain.

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References

1. Aamir, U.B., Wernery, U., Ilyushina, N., Webster, R.G. (2007) Characterization of avian H9N2 influenza viruses from United Arab Emirates 2000 to 2003. Virology. 361: 45-55.
2. Alexander, D.J. (2007) Summary of avian influenza activity in Europe, Asia, Africa, and Australasia, 2002-2006. Avian Dis. 51: 161-166.
3. Banet-Noach, C., Panshin, A., Golender, N., Simanov, L., Rozanblut, E., Pokamunski,S., et al. (2007) Genetic analysis of nonstructural genes (NS1 and NS2) of H9N2 and H5N1 viruses recently isolated in Israel. Virus Genes, 2007. 34: 157-68.
4. Butt, K.M., Smith, G.J., Chen, H., Zhang, L.J., Leung, Y.H. (2005) Human infection with an avian H9N2 influenza A virus in Hong Kong in 2003. J Clin Microbiol. 43: 5760-5767.
5. Capua, I., Alexander, D.J. (2008) Ecology, epidemiology and human health implication of avian influenza viruses: why do we need to share genetic data? Zoonoses public health. 55: 2-15.
6. Dundon, W.G., Milani, A., Cattoli, G., Capua, I., (2006) Progressive truncation of the Non-Structural 1 gene of H7N1 avian influenza viruses following extensive circulation in poultry. Virus Res. 119: 171-176.
7. Dundon, W.G., Capua, I. (2009) A Closer Look at the NS1 of Influenza Virus. Viruses. 1: 1057-1072.
8. Greenspan, D., Palese, P., Krystal, M. (1988) Two nuclear location signals in the influenza virus NS1 nonstructural protein. J Virol. 62: 3020-3026.
9. Guan, Y., Shortridge, K.F. S., Krauss, R.G., Webster, R.G. (1999) Molecular characterization of H9N2 influenza viruses: were they the donors of the "internal" genes of H5N1 viruses in Hong Kong? Proc Natl Acad Sci. USA. 96: 9363-9367.
10. Guo, Y., Li, J., Cheng, X. (1999) Discovery of men infected by avian influenza A (H9N2) virus.

Zhonghua Shi Yan He Lin Chuang Bing Du Xue Za Zhi. 13:105-108.

11. Haghighat-Jahromi, M., Asasi, K., Nili, H., Dadras, H., Shooshtari, A.H. (2008) Coinfection of avian influenza virus (H9N2 subtype) with infectious bronchitis live vaccine. Arch Virol. 153:651-655.
12. Hale, B.G., Randall, R.E., Orti´n, J., Jackson, D. (2008a) The multifunctional NS1 protein of influenza A Viruses. J Gen Virol. 89: 2359-2376.
13. Hale, B.G., Barclay, W.S., Randall, R.E., Russell, R.J. (2008b) Structure of an avian influenza A virus NS1 protein effector domain. Virology. 378: 1-5.
14. Hale, B.G., Batty, I.H., Downes, C.P., Randall, R.E. (2008c) Binding of influenza A virus NS1 protein to the inter-SH2 domain of p85 suggests a novel mechanism for phosphoinositide 3-kinase activation. J Biol Chem. 283: 1372-1380.
15. Hale, B.G., Jackson, D., Chen, Y.H., Lamb, R.A., Randall, R.E. (2006) Influenza A virus NS1 protein binds p85b and activates phosphatidylinositol-3-kinase signaling. Proc Natl Acad Sci USA. 103: 14194-14199.
16. Hawkins, P.T., Anderson, K.E., Davidson, K., Stephens, L.R. (2006) Signalling through Class I PI3Ks in mammalian cells. Biochem Soc Trans. 34: 647-662.
17. Iqbal, M., Yaqub, T., Reddy, K., W.McCauley, J. (2009) Novel Genotypes of H9N2 Influenza A Viruses Isolated from Poultry in Pakistan Containing NS Genes Similar to Highly Pathogenic H7N3 and H5N1 Viruses. PLos One. 4: e5788.
18. Jackson, D., Hossain, M.J., Hickman, D., Perez, D.R., Lamb, R.A. (2008) A new influenza virus virulence determinant: the NS1 protein four C-terminal residues modulate pathogenicity. Proc Natl Acad Sci USA. 105: 4381-4386.
19. Kendal, A.P., Pereira, M.S., Skehel, J.J. (1988) Concepts and Procedures for Laboratory-Based Influenza Surveillance. US department of health and human services. Public health service, centers for disease control, Atlanta, Georgia, USA.
20. Lee, C.W., Song, C.S., Lee, Y.J., Mo, I.P., Garcia. M., Suarez, D.L., et al. (2000) Sequence analysis of the hemagglutinin gene of H9N2 Korean avian influenza viruses and assessment of the pathogenic potential of isolate MS96. Avian Dis. 44: 527-35.
21. Long, J.X., Peng, D.X., Liu, Y.L., Wu, Y.T., Liu, X.F. (2008) Virulence of H5N1 avian influenza virus enhanced by a 15-nucleotide deletion in the viral nonstructural gene. Virus Genes. 36: 471-478.
22. Ludwig, S., Schultz, U., Mandler, J., Fitch, W.M., Scholtissek, C. (1991) Phylogenetic relationship of the nonstructural (NS) genes of influenza A viruses. Virology. 183: 566-577.
23. Lye, D.C., Nguyen, D.H., Giriputro, S., Anekthananon, T., Eraksoy, H., Tambyah, P.A. (2006) Singapore. Med J. 47: 471.
24. Macken, C.A., Webby, R.J., Bruno, W.J. (2006) Genotype turnover by reassortment of replication complex genes from avian influenza A virus. J Gen Virol. 87: 2803-2815.
25. Marion, R.M., Aragon, T., Beloso, A., Nieto, A., Ortin, J. (1997a) The N-terminal half of the influenza virus NS1 protein is sufficient for nuclear retention of mRNA and enhancement of viral mRNA translation. Nucleic Acids Res. 25: 4271-4277.
26. Matrosovich, M.N., Matrosovich, T.Y., Gray, T., Roberts, N.A., Klenk, H.D. (2004) Proc Natl Acad Sci USA. 101: 4620.
27. Naeem, K., Ullah, A., Manvell, R.J., Alexander, D.J. (1999) Avian influenza subtype H9N2 in poultry in Pakistan. Vet Rec. 145: 560.
28. Nemeroff, M.E., Barabino, S.M., Li, Y., Keller, W., Krug, R.M. (1998) Influenza virus NS1 protein interacts with the cellular 30 kDa subunit of CPSF and inhibits 39 end formation of cellular premRNAs. Mol Cell. 1: 991-1000.
29. Nili, H., Asasi, K. (2002) Natural cases and experimental study of H9N2 avian influenza in commercial broiler chickens of Iran. Avian Pathol. 31: 247-252.
30. Obenauer, J.C., Denson, J., Mehta, P.K., Su, X., Mukatira, S., Finkelstein, D.B., et al. (2006). Large-scale sequence analysis of avian influenza isolates. Science. 311: 1576-1580.
31. Palese, P., Shaw, M.L. (2007) Orthomyxoviridae: the viruses and their replication. In: Fields Virology, Knipe, D.M., Howley, P.M. (eds.). (5^{th} ed.). Philadelphia. Lippincott Williams & Wilkins, USA. p. 1647-1689.
32. Peiris, M. (1999) Influenza A H9N2: aspects of laboratory diagnosis. J Clin Microbiol. 37: 3426-7.
33. Salvatore, M., Basler, C.F., Parisien, J.P., Horvath, C.M., Bourmakina, S., Zheng, H., et al. (2002) Effects of influenza A virus NS1 protein on protein expression: the NS1 protein enhances translation and

is not required for shutoff of host protein synthesis. J Virol. 76: 1206-1212.
34. Tamura, K., Dudley, J., Nei, M., Kumar, S. (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol. 24: 1596-9.
35. Treanor, J.J., Snyder, M.H., London, W.T., Murphy, B.R. (1989) The B allele of the NS gene of avian influenza viruses, but not the A allele, attenuates a human influenza A virus for squirrel monkeys. Virology. 171: 1-9.
36. Vasfi Marandi, M., Bozorgmehri Fard, M.H. (2002) Isolation of H9N2 subtype of Avian Influenza Viruses during an outbreak in chickens in Iran. Iranian Biomed. J. 6: 13-17.
37. Webster, R.G., Bean, W.J., Gorman, O.T., Chambers, T.M., Kawaoka, Y. (1992) Evolution and ecology of influenza A viruses. Microbiol Rev. 56: 152-179.
38. Xu, K.M., Li, K.S., Smith, G.J., Li, J.W., Tai, H. (2007) Evolution and molecular epidemiology of H9N2 influenza A viruses from quail in southern China, 2000 to 2005. J Virol. 81: 2635-2645.
39. Zohari, S., Gyarmati, P., Thoren, P., Czifra, G., Brojer, C., Belak, S., et al. (2008) Genetic characterization of the NS gene indicates cocirculation of two sub-lineages of highly pathogenic avian influenza virus of H5N1 subtype in Northern Europe in 2006. Virus Genes. 36: 117-125.

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مطالعه مولکولی ژن‌های غیر ساختمانی (NS) ویروس آنفلوانزای طیور تحت تیپ H9N2 جدا شده از گله‌های گوشتی ایران

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چکیده

زمینه مطالعه: تحت تیپ H9N2 ویروس آنفلوانزای طیور در تمام کشورهای آسیایی و خاورمیانه پراکنده‌شده و در سال های اخیر مشکلات عدیده‌ای را در صنعت طیور ایران ایجاد کرده است. **هدف:** مطالعه ژن های موثر در بیماریزایی و تشخیص تحت تیپ H9N2 در کنترل شیوع آنفلوانزای طیور مهم می باشد. ژن غیر ساختمانی (NS) و پروتئین های حاصل از آن (NS1, NS2) در چرخه‌ی زندگی، حدت ویروس و همچنین تمایز گله‌های آلوده از واکسینه دخیل است. **روش کار:** ژن NS پنج جدایه از ویروس های آنفلوانزای تحت تیپ H9N2 جدا شده در خلال سال های ۱۹۹۸ تا ۲۰۱۰، از جهت توالی نوکلئوتیدی و اسیدآمینه‌ای کاملاً مورد بررسی قرار گرفتند. **نتایج:** ژن NS تمام این جدایه ها واجد ۸۹۰ نوکلئوتید و پروتئین NS1 تمامی آنها حاوی ۲۳۰ اسیدآمینه بود. در بین تمام جدایه های ایرانی موجود در بانک ژنی، تنها دوسویه واجد ۲۱۷ اسیدآمینه بود. بر اساس مطالعات فیلوژنتیکی ژن NS1 تمام جدایه های ایرانی موجود در بانک ژن و ۵ جدایه مطالعه‌شده در این مقاله، به دو زیر گروه I و II تقسیم شدند و مشخص شد از سال ۲۰۰۳ ژن NS جدایه های تحت تیپ H9N2 ایران از تحت تیپ های H7N3 پاکستان و از سال ۲۰۰۸ از تحت تیپ های H9N2 پاکستان نشات گرفته‌اند. **نتیجه‌گیری نهایی:** اگر چه ویروس های آنفلوانزای تحت تیپ H9N2 با زیر گروه I، از سال ۱۹۹۸ تا کنون در کشور ما در حال چرخش است ولی به نظر می رسد از سال ۲۰۰۳ زیر گروه II در حال چرخش در کشور می باشد که این تغییر می تواند به دلیل فشار واکسیناسیون و استفاده از سویه های زیر گروه I به عنوان سویه واکسن باشد و یا می تواند به دلیل باز آرایی ژنتیکی در جمعیت طیور بومی کشور باشد.

واژه‌های کلیدی: ویروس آنفلوآنزای پرندگان، تحت تیپ H9N2، ژن غیرساختمانی، طیور

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