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Phylogenetic analysis of NS gene of avian influenza viruses (H9N2) isolated from chicken in Iran during 2007–2011

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Abstract: To determine sequence characteristics and phylogenetic relationships of the nonstructural (NS) gene, the NS genes of 9 avian influenza (AI) viruses were sequenced and compared with other Iranian AI H9N2 viruses available in GenBank. The similarity among the earliest Iranian H9N2 isolate (A/chicken/Iran/ZMT-101/1998) and recent (2007–2011) studied isolates was low (94%). Some of the studied viruses revealed substitutions in RNA-binding and effector domains as well as antigenic regions of the NS protein. Recently studied isolates have a high similarity (97.4%–98.7%) with some Pakistani isolates (like A/chicken/Sawabi/NARC-2434/2006) in the NS gene. In the phylogenetic analysis, recently studied isolates composed a cluster separate from other Iranian isolates and were placed in the neighborhood of some Pakistani isolates. Hence, new Iranian H9N2 isolates may originate from Pakistani isolates. The NS protein of the Iranian H9N2 isolates differed from that of the Iranian highly pathogenic H5N1 viruses. The potential risk of the emergence of a pandemic virus because of the reassortment of the internal genes between H9N2 viruses and highly pathogenic AI viruses on one hand and the genetic and amino acid changes of AI viruses affecting their pathogenicity and immunogenicity on the other hand confirm the importance of continued surveillance of AI viruses.

Key words: Avian influenza, Iran, nonstructural gene, phylogenetic analysis

1. Introduction

The influenza A virus is a member of the family *Orthomyxoviridae*. Outbreaks of the H9N2 subtype of avian influenza (AI) in poultry of Asia and the Middle East started in the 1990s and continue to date (1). The AI H9N2 virus is now circulating on most continents of the world, including North America, Europe, Africa, and Asia (2). The outbreak of that subtype was also reported in Iran in 1998 and it has caused great economic losses for poultry farms. A catastrophic 65% mortality rate has been reported from some broiler farms in Iran (3). Although the H9N2 subtype has been considered as lowly pathogenic, it has the potential to cause severe respiratory disease with high mortality and decrease in egg production for poultry farms, especially in combination with other respiratory viruses and bacteria, such as infectious bronchitis, *Staphylococcus aureus*, *Avibacterium paragallinarum*, *Escherichia coli*, and immunosuppressive agents (4).

Segment 8 is the shortest part of the influenza virus RNA and has 890 nucleotides. It encodes for 2 nonstructural proteins: NS1 and NS2 (now called nuclear export protein) (5).

The NS1 protein is composed of variable number of amino acids (6). It is only expressed in influenza A virus-infected cells and is not present in mature virions (7). Because of the presence of the NS1 protein only in infected cells, antibody responses against this protein can be used as a marker of distinction between active infection and vaccination with killed vaccines in the chicken host (DIVA system) (8,9).

The NS1 protein is multifunctional and plays an important role as a posttranscriptional regulator in the influenza virus life cycle. It has thus been a focus of interest for many researchers.

There are 2 functional domains in the NS1 protein: the RNA-binding domain (residues 1–73) and the effector domain (residues 73–237), which mainly interacts with the host cell proteins (10). The bases responsible for binding to RNA in the RNA-binding domain are T5, P31, D34, R35, R38, K41, G45, R46, and T49 (11). The effector

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domain is responsible for interactions with many viral and cellular factors, including the virus polymerase and ribonucleoprotein (RNP) (6,10).

The NS1 protein is synthesized in the cytoplasm and is then transported and collects in the nucleus of the host cell (6). Its polypeptide contains the information needed for nuclear localization. There are 2 separate nuclear localization signals (NLS1 and NLS2) in the NS1 protein (12). The NLS1 amino acid sequence is highly conserved in all influenza A viruses and contains a sequence of D-R-L-R-R in residues 34–38. The NLS2 sequence is present within residues 203–237 (usually in residues 216–221) (13). Additionally, a leucine-rich sequence in residues 138–147 of the NS1 protein mediates the nuclear export of the protein. The nuclear export signal is inhibited by the neighbor amino acid sequence in the effector domain (12).

The NS1 protein helps to protect the influenza virus against the cellular cytokines (14). One of the significant functions of NS1 is to antagonize IFN-α/β-mediated antiviral response (12).

The NS2 protein is 121 amino acids in length and about 130–200 molecules of it have been detected in a mature virion (2). Less information is known about the NS2 protein functions. The NS2 protein mediates the export of newly synthesized RNPs from the nucleus to the cytoplasm and interacts with the viral matrix protein (M1) and cellular exportins (15).

The phylogenetic analysis of the nonstructural (NS) genes of different influenza viruses classified them into 2 groups: alleles A and B. The human, swine, most equine, and many avian influenza isolates are in the allele A group and one equine and many avian influenza isolates are in the allele B group (6).

The results of a recent study on Pakistani H9N2 isolates revealed that the NS genes of some new Pakistani H9N2 viruses had changed significantly compared with previous H9N2 viruses. There was evidence for reassortment of the NS gene from H9N2 viruses and HPAI H5N1 and H7N3 viruses (16). The potential consequences of this new genotype could be very important, especially from the point of view of the zoonotic aspects of H9N2 viruses. The occurrence of such a reassortment in Iranian H9N2 isolates is definitely probable.

In this study, the NS genes of 9 AI H9N2 strains isolated from poultry farms in different parts of Iran during 2007–2011, reference H9N2 strain A/chicken/Iran/N101/2011(H9N2), and other reported Iranian H9N2 isolates available in GenBank were genetically analyzed and nucleotide changes are discussed.

2. Materials and methods

2.1. Virus isolation

The strains A/chicken/Iran/N101/2011(H9N2), A/chicken/Iran/N102/2011(H9N2), A/chicken/Iran/N103/2011(H9N2), A/chicken/Iran/N104/2011(H9N2), A/chicken/Iran/ZMT-101/1998(H9N2), A/chicken/Iran/EBGV-86/2007(H9N2), A/chicken/Iran/EBGV-87/2008(H9N2), A/chicken/Iran/EBGV-88/2010(H9N2), and A/chicken/Iran/EBGV-89/2010(H9N2) were isolated from broiler farms affected by respiratory disease complex in different regions of Iran during 1998–2011. Tissue samples including trachea, lungs, and intestines were used for virus isolation. Initial isolation was performed in 10-day-old embryonated chicken eggs (ECEs).

2.2. Virus identification

Subtype identification of the virus was determined by standard hemagglutination-inhibition using specific H9 chicken antisera (Istituto Zooprofilattico Sperimentale delle Venezie, Padua, Italy) (17). Subtype identification of the virus was also determined by PCR tests specific for H9 and N2. The amplification protocol for H9 was: 1 step of denaturation at 95 °C for 2 min, followed by 40 cycles of denaturation at 95 °C for 1 min, annealing at 50 °C for 1 min, and extension at 72 °C for 1 min with 1 step of final extension at 72 °C for 10 min (18). The amplification protocol for N2 was: 1 step of denaturation at 95 °C for 2 min, followed by 35 cycles of denaturation at 95 °C for 1 min, annealing at 56 °C for 1 min, and extension at 72 °C for 1.5 min, with 1 step of final extension at 72 °C for 10 min (19).

Allantoic fluid was harvested from ECEs and used as stock virus for further analysis. All of the other H9N2 viral sequences of NS genes were present in GenBank.

2.3. RNA extraction and RT-PCR

Viral RNA was obtained from allantoic fluid by RNX Plus Kit (CinnaGen, Iran), according to the manufacturer's instructions. Reverse transcription was done by using influenza universal primer Uni12: 5'- AGCAAAAGCAGG -3' (20). Amplification of the NS full-length gene was carried out by PCR with specific primers (9,21).

The amplification protocol for the NS gene with NS1F1 and NS1R1 primers was: 1 step of denaturation at 95 °C for 2 min, followed by 35 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min with 1 step of final extension at 72 °C for 10 min. The amplification protocol for the NS gene with NS1F2 and NS1R2 primers was: 1 step of denaturation at 95 °C for 2 min, followed by 35 cycles of denaturation at 95 °C for 1 min, annealing at 50 °C for 1 min, and extension at 72 °C for 1.5 min with 1 step of final extension at 72 °C for 10 min. The reaction mixture was composed of 5 µL of cDNA, 2 µL of forward and reverse primers (each 20 pmol), and 25 µL of PCR master mix (CinnaGen) for both PCR tests. All used primer sequences are available in Table 1.

2.4. Gene sequence

PCR products were subjected to electrophoresis in a 1% (w/v) agarose gel. DNA fragments of the expected

Table 1. Primer sets used for RT-PCR in this study.

Gene	Forward primer	Reverse primer	Size (bp)
H9	P-H9-F: 5'-TTGCACCACACAGAGCACAAT-3'	P-H9-R: 5'-TGATGTATGCCCCACATGAA-3'	432
N2	P-N2-F: 5'-AGCAAAAGCAGGAGTGAAAATGAA-3'	P-N2-R: 5'-TTCTAAAATTGCGAAAGCTTATAT-3'	1447
NS	NS1F1: 5'-GCCGGAATTCATGGATTCCAACACTGTG -3'	NS1R1: 5'-GCCGCTGCAGCTACTTTGGAGAGAGTGT-3'	654
NS	NS1F2: 5'-AGCAAAAGCAGGGTG-3'	NS1R2: 5'-AGTAGAAACAAGGGTGTTTT-3'	890

H9: Hemagglutinin subtype 9, N2: neuraminidase subtype 2, NS: nonstructural.

length were extracted and purified with the GeneJET Gel Extraction Kit (Fermentas, Canada, catalog #k0691). The purified DNA fragments were cloned into the pTZ57R/T cloning vector (Fermentas, catalog #k1213). Three clones of each fragment were sequenced using M13 forward and M13 reverse primers (Promega, USA, catalog #Q5391 and #Q5401) at Sequetech Co. Ltd., USA. The sequences determined in this study are available in GenBank (Table 2).

2.5. Sequence analysis and phylogenetic study

Nucleotide and deduced amino acid sequences were edited with the EditSeq program, version 5 (DNASTAR Inc., USA). Using the studied sequences and some other H9N2 NS sequences available in GenBank, pairwise sequence alignments were performed with the Clustal W alignment algorithm, using the MegAlign program, version 5 (DNASTAR Inc.). Sequence similarity and phylogenetic relationships of different H9N2 subtype viruses were also studied with the MegAlign program.

3. Results

3.1. Analysis of nucleotide and amino acid sequences of the NS gene

The homology among NS nucleotide sequences from the 9 isolates sequenced in this study was 94%–99.8%. The similarity between the earlier (A/chicken/Iran/ZMT-101/1998) and recently studied isolates was relatively low (94%). Similarities among NS1 proteins of the 9 studied isolates were 90%–100%. The NS1 protein of recently studied isolates (2008–2011) differed by as much as 6.5%–13.9% from the earlier viruses isolated in the first years of the introduction of AI virus H9N2 to Iran. Similarities among different Iranian H9N2 isolates in the NS gene are shown in Table 3.

The NS1 protein of the studied isolates contained no amino acid deletions at positions 80–84. Amino acid substitutions in the NS1 protein of studied isolates were detected in 14.7% (34 of 230) positions. Most of

Table 2. Iranian influenza H9N2 isolates sequenced in this study.

No.	Virus	Accession number
1	A/chicken/Iran/N101/2011	KC428408
2	A/chicken/Iran/N102/2011	KC428406
3	A/chicken/Iran/N103/2011	KC428407
4	A/chicken/Iran/N104/2011	KC428409
5	A/chicken/Iran/EBGV-86/2007	JQ364986
6	A/chicken/Iran/EBGV-87/2008	JQ364987
7	A/chicken/Iran/EBGV-88/2010	JQ364984
8	A/chicken/Iran/EBGV-89/2010	JQ364988
9	A/chicken/Iran/ZMT-101/1998	JQ364985

Table 3. Comparison of homology (%) of the nucleotide and amino acid sequences of NS1 of different subgroups of Iranian isolates.

nc		First subgroup First cluster		Second subgroup				A/chicken/Hong Kong/610/79	
				Second cluster					
		nc	pr	nc	pr	nc	pr	nc	pr
First subgroup		91.3–99.6	87–100	88.3–95.6	86.1–94.8	90.1–95.2	83–94	93.7–97.7	90.4–98.7
Second subgroup	First cluster	–	–	94.6–99.6	93.1–99.6	92.4–96.9	88.9–97	92.5–95.7	92.2–96.1
	Second cluster	–	–	–	–	94.9–99.8	92.2–100	94–95.2	91.3–95.2

nc: Nucleotide, pr: protein.

the substitutions were localized in the region between positions 60 and 80, 124 and 145, and 200 and 220. For the NS2 protein, amino acid substitutions were detected in 7.4% (9 of 94) positions in Iranian isolates (Figure 1).

3.2. Analysis of nucleotide and amino acid sequences of the NS gene of the studied isolates in comparison with reference AI viruses

The NS gene of studied Iranian isolates did not have high similarities with prototype H9N2 strains A/quail/Hong Kong/G1/1997, A/duck/Hong Kong/Y280/1997, and A/chicken/Korea/MS96-CE6/1996 (the Korean H9N2 prototype strain) (89.1%–92.5%, 88.3%–91.9%, and 92%–95.5%, respectively) (Table 4). The NS1 gene and protein of A/chicken/Iran/ZMT-101/1998 shared high homologies with H9N2 strain A/chicken/Hong Kong/610/1979 (97.6% and 98.7%, respectively). Recently studied isolates exhibited less homology (91.3%–95.4%) with that strain (Table 4). The NS gene of Iranian isolates has high similarities to a H7N3 mallard isolate from Italy (A/mallard/Italy/43/2001) (93.1%–98.4%). The similarities were particularly high between the Italian H7N3 strain and earlier Iranian viruses isolated during 1998–1999 (>97%). An interesting result is the high homology (96.6%–97%) of some of the recently studied isolates (A/chicken/Iran/EBGV-88/2010 and A/chicken/Iran/EBGV-89/2010) with Indian H9N2 strain A/chicken/Orissa/2317/2004. However, some of the studied isolates (A/chicken/Iran/N101/2011, A/chicken/Iran/N102/2011, A/chicken/Iran/N103/2011, A/chicken/Iran/N104/2011) have lower similarity with that strain (91%) (Table 4). The recent studied isolates have a close relationship with some Pakistani isolates (A/chicken/Sawabi/NARC-2434/2006) (97.8%–98.7%) (Table 4).

3.3. Amino acid substitutions in the functional regions of the NS1 protein

In bases responsible for binding to RNA in the RNA-binding domain, all Iranian H9N2 viruses, including the studied isolates, contained R38 and K41. Furthermore, amino acid residues T5, P31, D34, R35, G45, R46, and T49, which mediate the NS–ds RNA interaction (12), were conserved in most of the Iranian isolates. However, some isolates had some substitutions in the RNA-binding domain.

The NLS1 motif (-D34-R35-L36-R37-R38-) was conserved among most of the Iranian H9N2 isolates (Table 5). Some of the studied viruses revealed substitution of R220W in the NLS2 motif. In positions 92 and 149 of NLS2, all Iranian H9N2 isolates, including the studied isolates, showed no substitution (Table 5).

In addition, some differences were found in the polyadenylation specificity factor (CPSF)-binding site, nuclear export signal sequences, and poly A binding protein II (PABII)-binding site among the studied viruses (Table 5). In the PDZ-binding motif, the 9 studied viruses contained PL motifs ESEV, KSEV, and KSEI. The ESEV motif was seen in earlier viruses isolated in 1998–1999. The KSEI motif came into existence in recent isolates after 2008 (Table 5).

Two potential immunodominant antigenic regions (IARs) have been reported for the NS1 protein: the first located at positions 53–80 and the second at positions 94–101 (9). Three amino acid substitutions were observed in the first IAR in most recently studied isolates (A60E, K62R, and E72K). In the second IAR, no amino acid substitution was observed in the studied isolates.

3.4. Genetic variability among the studied isolates

Analysis of the NS1 protein sequence variability showed that all of the Iranian isolates were distributed in 2 subgroups: the first subgroup contained viruses with K62, T91, I124, N127, N143, and E227; the second subgroup contained viruses with typical amino acids, R62, S91, H124, T127 (with 1 exception that contained alanine at this position), T143 (with 3 exceptions that contained alanine at this position), E/Y171 (glutamic acid or tyrosine), and K227. The studied viruses contained a separate cluster in the second subgroup. This cluster contained E60, K72 (1 isolate contained glutamic acid at this position), Y171, and N205 (Figure 1).

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Figure 1. Amino acid sequences of NS1 protein of the 9 studied H9N2 influenza virus isolates were compared. Residues identical to those of the A/chicken/Iran/zmt-101/98 virus are shown by dots and different residues are written.

I	A	S	V	P	A	S	R	Y	L	S	D	M	T	L	E	E	M	S	R	
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Figure 1. (Continued).

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Figure 1. (Continued).

Table 4. Homology (%) of the nucleotide and amino acid sequences of NS1 of the isolates sequenced in this study and H9N2 and H5N1 strains.

Virus	A/Qa/HK/G1/97 (H9N2)		A/Dk/HK/Y280/97 (H9N2)		A/Ck/Kor/MS96-CE6/96 (H9N2)		A/Ck/HK/610/79 (H9N2)		A/Ck/Orissa/2317/04 (H9N2)		A/Ck/Sawabi/NARC-2434/06 (H9N2)		A/Ck/Iran/53-3/08 (H5N1)	
	N	P	N	P	N	P	N	P	N	P	N	P	N	P
A/Ck/Iran/N101/2011	89.6	88.5	88.8	88.5	92.5	90.8	94.5	95.4	91.1	89.4	97.5	96.8	89.2	88.0
A/Ck/Iran/N102/2011	89.1	87.6	88.3	87.6	92	89.9	94	94.5	90.9	88.5	97.4	96.3	89.1	87.6
A/Ck /Iran/N103/2011	89.2	87.6	88.5	87.6	92.2	89.9	94.2	94.5	91.1	88.5	97.5	96.3	89.2	87.6
A/Ck /Iran/N104/2011	89.4	87.6	88.6	87.6	92.3	89.9	94.3	94.5	90.9	88.5	97.4	96.3	89.1	87.6
A/Ck /Iran/EBGV-86/2007	90.1	87.3	90.1	85.6	92.9	89.6	95.1	94.3	97.3	96.3	96.7	94.3	92.2	89.3
A/Ck /Iran/EBGV-87/2008	89.4	83.8	89.2	82.5	92.4	87.4	94.3	91.3	95.5	92.7	97.8	96.1	91	86.7
A/Ck /Iran/EBGV-88/2010	90.5	87.3	90	86	93.1	90	95.2	94.8	97	95.4	98.7	98.7	92	89.7
A/Ck /Iran/EBGV-89/2010	90.3	87.3	89.8	86	92.8	90	95	94.8	96.7	95.4	98.4	98.7	91.7	89.8
A/Ck /Iran/ZMT-101/1998	92.5	90.8	91.9	90	95.5	93.9	97.6	98.7	96.2	95.9	96.1	93.9	93.8	92.4

Ck: Chicken, Qa: quail, Dk: duck, N: nucleotide, P: protein.

Table 5. Amino acid substitutions in the functional regions of NS1 protein in Iranian isolates.

		NLS1 (34–38) 216–221	NLS2		NES (139–146)	CPSF-BS (103–106)	PABII-BS (216–230)	PDZ-ligand (227–230)
			92	149				
First subgroup		DRLRR*	PKQKRK	D	A	DRLENLIL	FMLM	PKOKRKMARTIESEV	ESEV EPEV
Second subgroup	First cluster	. . . . .*	. . . . . .*	D	A	. . . DT. .	. . . .	. . . . . .K. . .*	KSEV
	Second cluster	. . . . .	. . . . W .	D	A	. . . DT. . . . . ET. .	. . . .	. . . . W. . . . . .K. . .	KSEI

*: With some exceptions. NLS: Nuclear localization signal, NES: nuclear export signal, CPSF-BS: polyadenylation specificity factor, PABII-BS: poly (A) binding protein II, PDZ-PDZ binding domain.

3.5. Phylogenetic analysis

The NS genes of the studied isolates, 27 other Iranian H9N2 isolates, and reference H9N2 strains available in GenBank were used for phylogenetic analysis (Figures 2 and 3). The group of Iranian H9N2 isolates was divided into 2 subgroups. The first subgroup contained viruses isolated mainly before 2004. The second subgroup contained isolates isolated mainly after 2004. The second subgroup also subdivided into 2 clusters: the first cluster was composed of viruses isolated mainly before 2008 and the second cluster was composed of viruses isolated after 2008. Almost all isolates sequenced in this study were related to the second cluster (Figure 2). NS1 genes of all the AI viruses isolated in Iran were located in allele A (Figure 3).

The second phylogenetic analysis was related to the position of NS1 genes of Iranian H9N2 isolates among AI viruses isolated in Asia, the Middle East, and other regions.

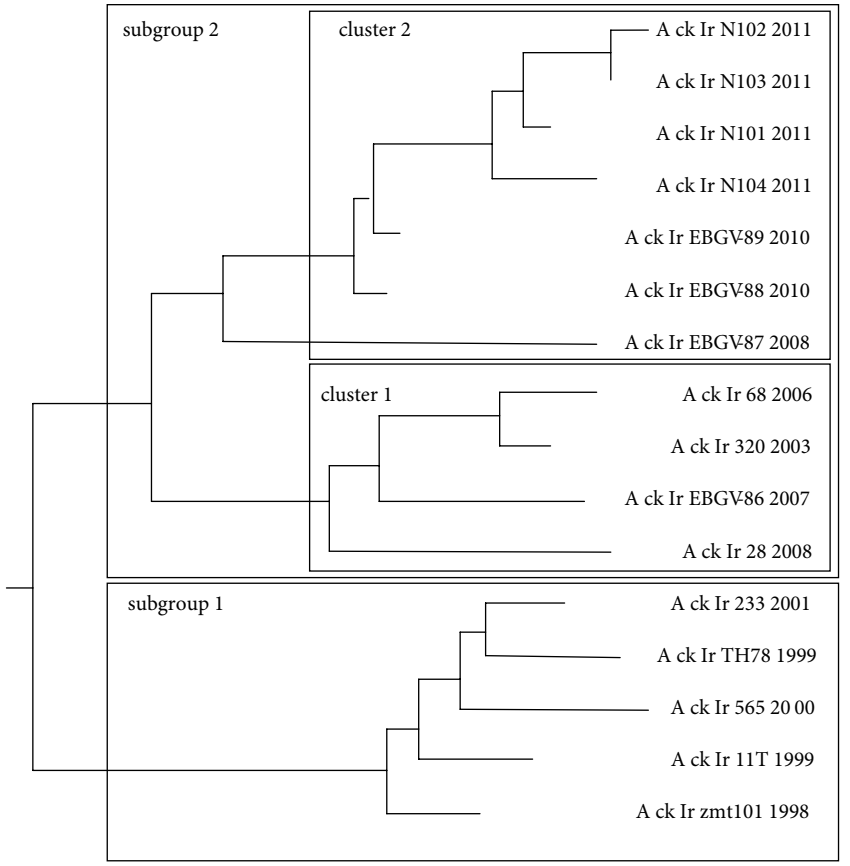

Figure 2. Phylogenetic analysis of Iranian isolates; trees were generated by the Clustal W method with MegAlign software. Iranian H9N2 AI viruses classified in different subgroups and clusters are shown in boxes. The length of horizontal lines is proportional to the minimum nucleotide differences needed to join the nodes. Vertical lines are used just for spacing branches and labels. All viruses sequenced in this study and the other sequences can be found in GenBank. Ck: Chicken, Qa: quail, Dk: duck, Ir: Iran. Subgroup 1: A/chicken/Iran/233/2001 (HQ333526), A/chicken/Iran/TH78/1999 (FJ205647), A/chicken/Iran/565/2000 (HQ333529), A/chicken/Iran/11T/99 (AF508710), A/chicken/Iran/ZMT-101/1998 (JQ364985). Subgroup 2, cluster 1: A/chicken/Iran/68/2006 (HQ333524), A/chicken/Iran/320/2003 (HQ333527), A/chicken/Iran/TH186/2007 (FJ205652), A/chicken/Iran/TH85/2006 (FJ205651), A/chicken/Iran/EBGV-86/2007 (JQ364986), A/chicken/Iran/28/2008 (HQ333523). Subgroup 2, cluster 2: A/chicken/Iran/N102/2011 (KC428406), A/chicken/Iran/N103/2011 (KC428407), A/chicken/Iran/N101/2011 (KC428408), A/chicken/Iran/N104/2011 (KC428409), A/chicken/Iran/EBGV-89/2010 (JQ364988), A/chicken/Iran/EBGV-88/2010 (JQ364984), A/chicken/Iran/EBGV-87/2008 (JQ364987).

Most of the recent Iranian isolates composed a separate cluster and were placed in the neighborhood of some Pakistani isolates (A/chicken/Sawabi/NARC-2434/2006 and A/chicken/Pakistan/UDL-03/2008). Furthermore, 1 of the studied isolates, A/chicken/Iran/EBGV-87/2008, was grouped in 1 cluster with the Pakistani isolates (Figure 3).

It was also revealed that A/quail/Dubai/303/2000 has a relationship with the Iranian group in the NS gene phylogenetic trees (Figure 3). According to phylogenetic analysis, the NS gene of Iranian isolates is different enough from A/quail/Hong Kong/G1/1997-like, A/duck/Hong Kong/Y280/1997-like, and Korean-like H9N2 isolates to be considered as a different sublineage (Figure 3). A significant point in the phylogenetic analysis is the close relationship between A/chicken/Hong Kong/610/1979 and the first subgroup of Iranian isolates (Figure 3).

4. Discussion

According to the results of nucleotide sequence homology, the NS gene has been divided into alleles A and B (6). The NS genes of the all Iranian isolates were classified as allele A (Figure 3).

The similarity between the NS gene of earlier (A/chicken/Iran/ZMT-101/1998) and recently studied isolates was about 94%. This low similarity may be because of different predecessors of these viruses. In fact, recent Iranian viruses may obtain their NS gene from another influenza virus of a different subtype by genetic reassortment. Such an assumption was proven for some recent Pakistani isolates. The results of a recent study on Pakistani H9N2 isolates revealed that the reassortment of NS gene has happened between viruses of H9N2 and HPAI H5N1 subtypes (16).

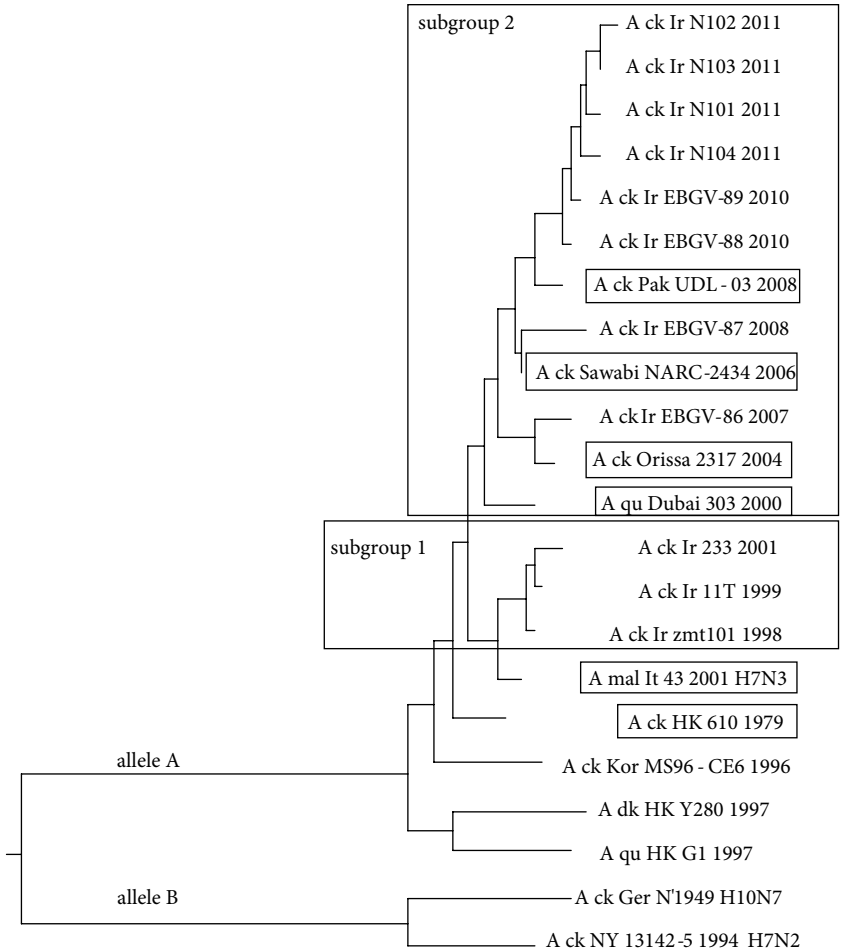

Figure 3. Phylogenetic analysis of Iranian isolates with other reference and neighbor-country isolates; trees were generated by the Clustal W method with MegAlign software. Iranian and other countries' H9N2 AI viruses classified in different subgroups are shown in boxes. Some H9N2 viruses mentioned in Section 3 are shown inside rectangles. The length of horizontal lines is proportional to the minimum nucleotide differences needed to join the nodes. Vertical lines are used just for spacing branches and labels. All viruses sequenced in this study and the other sequences can be found in GenBank. Alleles A and B are shown. Ck: Chicken, Qa: quail, Dk: duck, Mal: mallard, Ir: Iran, Pak: Pakistan, HK: Hong Kong, Kor: Korea, Ger: Germany, It: Italy. Allele A, subgroup 1: A/chicken/Iran/233/2001 (HQ333526), A/chicken/Iran/11T/99 (AF508710), A/chicken/Iran/ZMT-101/1998 (JQ364985). Allele A, subgroup 2: A/chicken/Iran/N102/2011 (KC428406), A/chicken/Iran/N103/2011 (KC428407), A/chicken/Iran/N101/2011 (KC428408), A/chicken/Iran/N104/2011 (KC428409), A/chicken/Iran/EBGV-89/2010 (JQ364988), A/chicken/Iran/EBGV-88/2010 (JQ364984), A/chicken/Iran/EBGV-87/2008 (JQ364987), A/chicken/Iran/28/2008 (HQ333523), A/chicken/Iran/EBGV-86/2007 (JQ364986). Allele A, other strains: A/chicken/Pakistan/UDL-03/2008 (CY038478), A/chicken/Sawabi/NARC-2434/2006 (JN540060), A/chicken/Orissa/2317/2004 (CY068655), A/quail/Dubai/303/2000 (EF063540), A/mallard/Italy/43/01 (H7N3) (AY586443), A/duck/Hong Kong/610/79 (AF523517), A/chicken/Korea/MS96-CE6/1996 (GU053190), A/Duck/Hong Kong/Y280/97 (AF156475), A/Quail/Hong Kong/G1/97 (AF156477). Allele B: A/chicken/Germany/N/1949(H10N7) (GQ176132), A/Chicken/NY/13142-5/94 (H7N2) (IAAF001409).

However, the NS protein of the Iranian H9N2 isolates differed completely from those of the Iranian highly pathogenic H5N1 viruses (Table 4). The possible reassortment of NS and other internal genes of Iranian H9N2 viruses should be checked for continuously. H9N2 viruses present potential public health risks as donors or recipients of the internal genes to or from H5N1 viruses. The substitution of the NS gene from the local H9N2 strain into local H5N1 viruses and vice versa could lead to unpredictable results in the pathogenicity of the created virus, especially for humans.

The NS gene of Iranian H9N2 isolates was not classified into groups of NS genes of famous prototype H9N2 viruses (A/quail/Hong Kong/G1/1997, A/duck/Hong Kong/Y280/1997, and Korean H9N2 isolates) (Figure 3). Israeli scientists have suggested that the H9N2 viruses that caused outbreaks in Israel and Jordan had received their NS1 genes from a A/duck/Hong Kong/610/1979 (H9N2)-

like virus (22). Phylogenetic analysis showed that A/duck/Hong Kong/610/1979 was also close to Iranian H9N2 viruses isolated during the first years of AI epizootic in Iran (Figure 3). Therefore, this virus could be the progenitor of the NS gene for early Iranian H9N2 isolates. The difference between the earliest isolate sequenced in this study (A/chicken/Iran/zmt-101/1998) and A/duck/Hong Kong/610/1979 was only 2.4% (Table 4).

Amino acid substitutions in the NS1 protein of the studied isolates were present in 14.7% of positions, whereas in the NS2 protein 7.4% of amino acids were substituted. This result confirms the higher conservation of the NS2 gene (6).

There are 2 functional domains in the NS1 protein: the RNA-binding domain (residues 1–73) and the effector domain (residues 73–237), which mainly interacts with the host cell proteins (10).

In the RNA-binding domain, the sequence of the NLS1 in AI viruses of allele A typically is -DRLRR- (6). The Iranian viruses also had that sequence.

The typical residue of the allele A viruses at position 143 is threonine. The first subgroup of Iranian isolates had asparagine and the second subgroup had threonine at this position (with some exceptions, in which alanine was substituted).

The NLS2 sequence was more variable than the NLS1 sequence. Most of the Iranian isolates had the NLS2 sequence of PKQKRK, which is typical of the viruses of allele A (6). The second cluster of Iranian isolates had NLS2 sequences of PKQKWK.

The pathogenicity of AI viruses may be associated with 2 sites (CPSF and PABII) located in the NS1 effector domain (23,24). Some of the studied Iranian isolates had some differences in these sites.

The other domain, which is responsible for protein interaction and transmission of the cellular signal in NS1 protein, is the PDZ domain (25). In the PDZ-binding motif, the 9 studied viruses contained PL motifs ESEV, KSEV, and KSEI. The ESEV motif was seen in earlier viruses isolated in 1998–1999. The KSEI motif came into existence in recent isolates after 2008 (Table 5). The EPEV motif, which was present in some of the Iranian H9N2 isolates in the first subgroup, is typical of the NS1 proteins of the virulent human H5N1 viruses (26). The insertion of 4 C-terminal amino acids, including ESEV, EPEV, or KSEV, into avirulent viruses increased virus virulence and caused severe disease signs in mice (27). The KSEI sequence in the PDZ domain, present in some studied isolates and some of the recent Pakistani H9N2 isolates (16), is a rare sequence. Obenauer et al. (25) showed that isoleucine at the C-terminal position and lysine at the –4 position in the NS1 protein of influenza viruses were rare. Lysine at position –4 was seen in the H1N1 1918 pandemic virus, 2 H5N1 viruses isolated during 2005 in Indonesia, and 2 H5N1 viruses isolated in 2007 from Saudi Arabia, which contained a KSEV C-terminal sequence (25,28).

Antigenic studies to date have detected 3 T-cell epitopes (13–32, 152–160, and 122–130 amino acid positions) (29,30) and 2 B-cell epitopes (42–53 and 206–215 amino acid positions) (31). In addition, predictive antigenic analysis of the NS1 protein revealed 2 potential IARs: amino acid positions 53–80 and 94–101 (9). Most of substitutions in the NS1 gene of the studied isolates were localized in the regions between positions 60 and 80, 124 and 145, and 200 and 220. Variable amino acid sites at positions 48, 60, 62, 72, 124, 126, and 127 of the NS1 protein of Iranian isolates occupied regions associated with the above-mentioned antigenic sites or potential IARs. Amino acid substitutions in epitopes of the NS1 protein could affect the accuracy of the DIVA system, based on detection of anti-NS1 antibodies, to differentiate between vaccinated and disease-affected flocks (9).

It was revealed that some amino acids have substitutions in functional regions of the NS1 gene of recently studied viruses compared to earlier isolates (Table 5). Understanding the influence of those substitutions on the pathogenicity of the AI virus requires further studies.

According to the phylogenetic analysis, the group of Iranian H9N2 isolates is divided into 2 subgroups and the second subgroup is also subdivided into 2 clusters. The subdivisions of Iranian isolates in phylogenetic analysis were in coordination with their times of isolation. The differences between these 2 subgroups were 4.4%–11.7% (Table 3). The differences between the 2 clusters of the second subgroup were 3.1%–7.6%. Phylogenetic analyses of Iranian isolates including those available in GenBank proposed that in around 2008 a new variant of the H9N2 virus entered Iranian poultry farms (Figure 2). Previously, it was showed that such subdivision was characteristic for the hemagglutinin gene, too (32). It is reasonable to hypothesize that the viruses of this cluster acquired an epidemiological advantage. It is probable that simultaneous changes in several genes conferred the adaptability to spread among poultry birds in different regions of Iran in the second cluster of the second subgroup of Iranian H9N2 isolates.

The viruses with NS1 protein of the second cluster of the second subgroup are now circulating in Iran. Significant similarity (97.4%–98.7%) was seen in the NS gene of most of the recent Iranian isolates and some recent Pakistani isolates (A/chicken/Sawabi/NARC-2434/2006 and A/chicken/Pakistan/UDL-03/2008) (Table 4). On the other hand, recently studied Iranian isolates composed a

separate cluster and were placed in neighborhood of the recent Pakistani isolates in the phylogenetic tree (Figure 3). These facts support the idea that recent Iranian H9N2 isolates (second cluster of the second subgroup; Figure 2) may originate from Pakistani isolates. Interestingly, the time of isolation of Pakistani isolates was earlier than the isolation of recent Iranian viruses.

In brief conclusion, the phylogenetic tree of the NS gene of Iranian H9N2 isolates showed that a new cluster of strains has been emerging in poultry flocks since 2008. This new cluster's viruses have some differences in the NS gene and protein in comparison to earlier isolates. These viruses could emerge because of the introduction of a new H9N2 virus from Pakistan or genetic reassortment between Iranian and Pakistani AI viruses. The results revealed that recently studied (2008–2011) Iranian isolates were placed in the neighborhood of some recent Pakistani isolates. However, the difference in the NS1 protein of the earlier and recent viruses is to some extent attributed to continued point mutations in earlier isolates because of the pressure of vaccination. Iranian AI vaccine strains are members of the first subgroup. In both conditions, changes in the important regions of the NS1 protein, as observed in some studied viruses, could affect different features of the virus.

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References

1. Alexander DJ. An overview of the epidemiology of avian influenza. Vaccine 2007; 25: 5637–5644.
2. Yasuda J, Nakada S, Kato A, Toyoda T, Ishihama A. Molecular assembly of influenza: association of the NS2 protein with virion matrix. Virology 1993; 196: 249–255.
3. Nili H, Asasi K. Avian influenza (H9N2) outbreak in Iran. Avian Dis 2003; 47: 828–831.
4. Bano S, Naeem K, Malik SA. Evaluation of pathogenic potential of avian influenza virus serotype H9N2 in chickens. Avian Dis 2003; 47: 817–822.
5. Lamb RA, Chopin PW, Chanock RM, Lai CJ. Mapping of the two overlapping genes for polypeptides NS1 and NS2 on RNA segment 8 of influenza virus genome. P Natl Acad Sci USA 1980; 77: 1857–1861.
6. Suarez DL, Perdue ML. Multiple alignment comparison of the non-structural genes of influenza A viruses. Virus Res 1998; 54: 59–69.
7. Lazarowitz SG, Compans RW, Choppin PW. Influenza virus structural and nonstructural proteins in infected cells and their plasma membranes. Virology 1971; 46: 830–843.
8. Tumpey TM, Alvarez R, Swayne DE, Suarez DL. Molecular assembly of influenza virus: association of the NS2 protein with virion matrix. J Clin Microbiol 2005; 43: 676–683.
9. Panshin A, Golender N, Davidson I, Nagar S, Garsia M, Jackwood MW, Mundt E, Alturi A, Perk S. Variability of NS1 proteins among H9N2 avian influenza viruses isolated in Israel during 2000–2006. Virus Gen 2010; 41: 396–405.
10. Lin D, Lan J, Zhang Z. Structure and function of the NS1 protein of influenza A virus. Acta Bioch Bioph Sinica 2007; 39: 155–162.
11. Yin C, Khan JA, Swapna GVT, Ertekin A, Krug RM, Tong L, Montelione GT. Conserved surface features from the double-stranded RNA binding site of non-structural protein 1 (NS1) from influenza A and B viruses. J Biol Chem 2007; 282: 20584–20595.
12. Hale BG, Randall RE, Ortin J, Jackson D. The multifunctional NS1 protein of influenza A viruses. J Gen Virol 2008; 89: 2359–2376.
13. Banet-Noach C, Panshin A, Golender N, Simanov L, Rozanblut E, Pokamunski S, Pirak M, Tendler Y, Garcia M, Gelman B et al. Genetic analysis of nonstructural genes (NS1 and NS2) of H9N2 and H5N1 viruses recently isolated in Israel. Virus Genes 2007; 34: 157–168.
14. Seo SH, Hoffmann E, Webster RG. Lethal H5N1 influenza viruses escape host anti-viral cytokine responses. Nat Med 2002; 8: 950–954.
15. O'Neill RE, Talon J, Palese P. The influenza virus NEP (NS2 protein) mediates the nuclear export of viral ribonucleoproteins. EMBO J 1998; 17: 288–296.
16. Iqbal M, Yaqub T, Reddy K, McCauley JW. 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 2009; 5: e5788.
17. Shortridge KF, Butterfield WK, Webster RG, Campbell CH. Isolation and characterization of influenza A viruses from avian species in Hong Kong. Bull WHO 1977; 55: 15–20.
18. Noroozian H, Vasfi Marandi M, Razazian M. Detection of avian influenza virus of H9 subtype in the faeces of experimentally and naturally infected chickens by reverse transcription-polymerase chain reaction. Acta Vet Brno 2007; 76: 405–413.
19. Cameron KR, Gregory V, Banks J, Brown IH, Alexander DJ, Hay AJ, Lin YP. H9N2 subtype influenza A viruses in poultry in Pakistan are closely related to the H9N2 viruses responsible for human infection in Hong Kong. Virology 2000; 278: 36–41.
20. Hoffmann E, Stech J, Guan Y, Webster RG, Perez DR. Universal primer set for the full-length amplification of all influenza A viruses. Arch Virol 2001; 146: 2275–2289.

21. Talazadeh F, Mayahi M, Seifi M, Pourmehdi M. Survey on ELISA based on anti influenza A NS1 antibodies to differentiate the infected and vaccinated poultries. Jundishapur J Microbiol 2013; 6: e7055.

22. Golender N, Panshin A, Banet-Noach C, Nagar S, Pokamunski S, Pirak M, Tendler Y, Davidson I, Garcia M, Perk S. Genetic characterization of avian influenza viruses isolated in Israel during 2000–2006. Virus Gen 2008; 37: 289–297.

23. Noah DL, Twu KY, Krug RM. Cellular antiviral responses against influenza A virus are countered at the post-transcriptional level by the viral NS1A protein via its binding to a cellular protein required for the 3' end processing of cellular pre-mRNAs. Virol 2003; 307: 386–395.

24. Twu KY, Noah DL, Rao P, Kuo R-L, Krug RM. The CPSF30 binding site on the NS1A protein of influenza A virus is a potential antiviral target. J Virol 80: 3957–3965.

25. Obenauer JC, Denson J, Mehta PK, Su X, Mukatira S, Finkelstein DB, Xu X, Wang J, Ma J, Fan Y et al. Large-scale sequence analysis of avian influenza isolates. Science 2006; 311: 1576–1580.

26. Zohari S, Gyarmati P, Ejdersund A, Berglöf U, Thorén P, Ehrenberg M, Czifra G, Belák S, Waldenström J, Olsen B et al. Phylogenetic analysis of the nonstructural (NS) gene of influenza A viruses isolated from mallards in Northern Europe in 2005. Virol J 2008; 5: 147.

27. Jackson D, Hossain M J, Hickman D, Perez DR, Lamb RA. A new influenza virus virulence determinant: the NS1 protein four C-terminal residues modulate pathogenicity. P Natl Acad Sci USA 2008; 105: 4381–4386.

28. Monne I, Fusaro A, Al-Blowi MH, Ismail NM, Khan OA, Dauphin G, Tripodi A, Salviato A, Marangon S, Capua I et al. Cocirculation of two sublineages of HPAI H5N1 virus in the Kingdom of Saudi Arabia with unique molecular signatures suggesting separate introductions into the commercial poultry and falconry sectors. J Gen Virol 2008; 89: 2691–2697.

29. Hackett CJ, Horowitz D, Wysocka M, Dillon SB. Influenza virus infection elicits class II major histocompatibility complex-restricted T cells specific for an epitope identified in the NS1 non-structural protein. J Gen Virol 1992; 73: 1339–1343.

30. Jameson J, Cruz J, Ennis FA. Human cytotoxic T-lymphocyte repertoire to influenza A viruses J Virol 1998; 72: 8682–8689.

31. Tan Z, Akerstrom S, Wee BY, Lal SK, Mirazimi A, Tan YJ. A new panel of NS1 antibodies for easy detection and titration of influenza A virus. J Med Virol 2010; 82: 467–475.

32. Norouzian H, Gholami SJ, Vasfi-Marandi M. Phylogenetic analysis of hemagglutinin gene of H9N2 avian influenza viruses isolated from chicken in Iran in 2010-2011: emerging of a new subgroup. Iranian J Virol 2012; 6: 18–26.