Molecular and phylogenetic analysis of hemagglutinin (HA) and neuraminidase (NA) genes of H9N2 avian influenza viruses isolated from vaccinated broiler chickens in Iran in 2011

Abstract

H9N2 avian influenza outbreaks have been one of the major problems of poultry industry in Iran. A vaccination program has been implemented to control disease since 1988, but some outbreaks continued to occur in several broiler flocks. In this study four strains which were isolated in 2011 from vaccinated broiler flocks with a history of respiratory illness were sequenced. Sequenca analysis HA and NA genes indicated that all strains isolated in this study shared nucleotide homologies of 91.3% to 92.4% and 90.4% to 91.1% with the vaccine strain for HA and NA genes, respectively. Extra potential glycosylation sites could be observed in HA and NA genes of current isolates versus vaccine strain. Molecular analysis of all four isolated strains showed KSSR motif of cleavage site in the HA genes, while it is RSSR for typical strains in Iran. Phylogenetic analysis of HA and NA genes showed that all strains isolated in this study fell into the same subgroup and they belong to A/Quail/HongKong/G1/97 H9N2 lineage but in comparison with vaccine strain they had some deviation in the decade and fell into different subgroup. Other changes included loss, addition and variation in predicted epitope in HA and NA genes of field strains. These results suggest that the commercial vaccine genetically is not enough coordinated with current viruses in region. Therefore,

constant evaluation of important genes induced immunity and vaccine efficiency in protection against these strains would be essential.

Key words: Phylogenetic analysis-Hemagglutinin-Neuraminidase-Influenza virus-Glycosylation-Epitope-Iran-Vaccine

Introduction

Avian influenza is caused by type A influenza virus and is categorized based on the activity of two surface glycoprotein; Hemagglutinin and neuraminidase(Webster et al., 1992; Guo et al., 2000). There are 16 HA and 9 NA subtypes responsible for causing infection. HA plays an essential role in the early stages of infection and is responsible for the virus binding to its receptor, sialic acid, which is present on the host cell surface and promotes fusion of viral and endosomal membranes and eventually facilitates viral entry into the host cell (Webster et al., 1992). The NA surface glycoprotein of influenza viruses prevents virus aggregation by cleaving the α-ketosodic linkage between sialic acid and adjacent sugar residue. This results in destruction of receptors recognized by HA and facilitates the to and fro movement of the virus from the site of infection (Gubareva et al., 2000; Guo et al., 2000). Avian influenza virus (AIV) H9N2 which is known to infect poultry populations throughout the world, is derived from the Eurasian and the North American influenza virus genes(Fouchier et al., 2005). In chickens More recently,

H9N2 viruses have been reported in Middle Eastern countries and have been responsible for widespread and serious disease problems in commercial chickens in Iran, Pakistan, Saudi Arabia and United Arab Emirates (Banks et al., 2000; Nili and Asasi. 2002; Vasfi Marandi and Bozorgmehri-Fard. 2002; Capua and Alexander. 2006; Nagarajan et al., 2009; Bozorgi et al., 2012)

The first report of AIV (H9N2) in poultry flocks in Iran was in 1998. Since 1998 H9N2 avian influenza outbreaks have been one of the major problems in Iranian poultry industry(Karimi et al., 2004). These highly contagious viruses were spreading in poultry flocks of other provinces of country, thus a vaccination strategy by using inactivated H9N2 vaccine based on A/Ck/Iran/av1221/98 was adopted to control AI disease in poultry industry (Vasfi Marandi and Bozorgmehri-Fard. 2002). In spite of using of this vaccine, some outbreaks continued to occur in several broiler flocks with great economic losses. All H9N2 subtypes isolated from vaccinated or unvaccinated chickens belonged to non HPAI till now(Vasfi Marandi and Bozorgmehri-Fard. 2002; Soltanialvar et al., 2010)

Present study was conducted to carry out phylogenetic analysis and molecular characterization of new strains isolated in Iran and establish the phylogenetic relationships between Iranian Isolates and neighboring or Eurasian sublineages since 1998 until 2011; also to identify and predict epitopes for antigenic changes and

variations in glycosylation sites, and to compare circulating viruses with vaccine strain yearly.

Material and Methods

Sampling and virus isolation: samples were collected from 25 farms in various parts of country since February until December 2011. Tissue samples usually included trachea and lung. All samples were collected from flocks with the history of acute respiratory illness and vaccination against H9N2 influenza virus. Virus isolation was carried out in 9-11 days old SPF embryonated eggs respecting the standard protocol (OIE,2005). Virus subtypes were determined by a standard Hemagglutination Inhibition (HI) and Neuraminidase Inhibition (NI) test (Shortridge et al,1997). The four isolated viruses in this study were named as follow: (A/chicken/Iran/186/2011,A/chicken/Iran/187/2011,A/chicken/Iran/189/2011,A/chicken/Iran/191/2011). The A/Ck/Iran/av1221/98 H9N2 virus which is the selected strain for producing vaccine in iran since 1998 was used for molecular and phylogenetic analysis.

RT-PCR and sequence analysis: The field viruses RNA were extracted directly from the allantoic fluid by means of High Pure viral Nucleic Acid Kit (Roche Germany).The RNA of vaccine strain was extract from a commercial vaccine in Iran.
Purified genomic RNA was used to generate cDNA clones by RT-PCR through the standard procedure which was using specific primers

Primers used for HA amplification were:

Forward primer (1720 bp): 5'-AGCAAAAGCAGGGG-3'

Reverse primer (1720 bp): 5'-AGTAGAAACAAGGGTGTT-3'

Primers used for NA amplification were:

Forward primer (1470 bp): 5'-GCAAAAGCAGGAGTGAAATGA-3'

Reverse primer (1470 bP): 5'-GGTTCTAAAATTGCGAAAGCC-3'

The PCR products were purified using High Pure product purification kit (Roche Germany). These products were applied to low Melting Point (LMP) agarose and distinct bands were purified from gel for sequencing (MWG,Germany).

Nucleotide and deduced amino acid sequences of HA and NA genes from new isolated strains were edited by BioEdit version 7. Nucleotid and amino acid sequences were aligned by ClustalW, In order to compare H9N2 viruses isolated in this study with circulating virus since 1998 to 2011, a computerized search of all available sequences of avian influenza Virus subtype H9N2 reported in poultry flocks in neighboring countries of Iran and the Eurasian G1 sublineage reference was conducted. The search was performed with National Centre for Biotechnology Information (NCBI) Flu Database(Bao et al., 2008) and sequences of NA and HA were retrieved and downloaded (data not shown).The phylogenetic analysis was performed with the Mega 5 program(Tamura et al., 2011)

Determination of glycosylation site

To determine glycosylation sites of viral strain, an online server application, ScanProsite (de Castro et al., 2006) was used to compare and identify N-glycosylation sites.

Epitope analysis of HA and NA for antigenic variations

Epitope prediction was performed by CTL (Cytotoxic T Lymphocyte) epitope prediction method (Bhasin and Raghava. 2004). The amino acid sequences of HA and NA proteins from selected strains were used in this step and the predicted antigenic sites from each sequence were compared so that composition similarities and differences were determined.

Phylogenetic analysis of HA and NA nucleotides.

Sequencing avian influenza H9N2 viruses isolated from Iran since 1998 until 2011 was done by MEGA 5 (Tamura et al., 2011).. The selected nucleotide sequences based on local alignment and homology searches using BLAST were aligned by using CLUSTALW. Phylogenetic trees were constructed by using neighbor-joining tree with maximum composite likelihood. Internal branching probabilities were determined by bootstrap analysis of 1000 replicates and were indicated by percentage value on each branch.

Results

Disease signs in the field: The symptoms included anorexia, reduced feed consumption, gasping and conjunctivitis with facial edema. The major post mortem findings were tracheitis, respiratory congestion. Mortality was up to 10%, resulting in considerable economic losses.

Analysis of HA and NA sequences of the H9N2 viruses

The sequenced part of the HA gene contains 1720 nucleotide coding 566 amino acid residues. This protein region includes a complete receptor-binding pocket, cleavage site, glycosylation site and various antigenic epitopes. In order to analyse them we compared the HA amino acid sequences (deduced from the nucleotide sequences) of new strains isolated in this study with those of other Iranian isolates between 1998 and 2011, vaccine strain and the Eurasian G1 sublineage reference strain (A/Quail/HongKong/G1/97).

Nucleotide sequence analysis of the new HA genes showed homologies of 92/2-99/6%and shared a homology of (90.8-92%) with H9N2 isolates of A/QA/HK/G1/97 lineage.

In this study, 1410 base pairs of the NA genes (ORF region) were sequenced and amino acid sequences (469) of the 4 isolates were deduced from the nucleotide sequence. The nucleotid sequence of the isolates were highly identical (95.4-99.6%) and shared a homology of (88.3 - 89%) with H9N2 isolates of A/QA/HK/G1/97 lineage.

The deduced amino acid sequence revealed that the HA cleavage site motif was

KSSR/GLF.

The receptor binding site motif

Analysis of the HA receptor binding pocket in particular showed that, except for A/Ck/Ir/584/20, A/Ck/Ir/TH81/02, A/Ck/Ir/TH81/02andA/Ck/Ir/119/05 isolates, other H9N2 isolates from Iran contained Leucine (L) at position 234(Table 1)

N-Glycosylation site of HA and NA

Potential glycosylation sites with the N-X-T/S motif (in which X may be any amino acid except proline) were predicted by ScanProsite for HA and NA viral proteins isolated from Iran shown in Tables 3 and 4 respectively. A total of seven predicted glycosylation sites (PGS) at position (29-32; 105-108; 141-144; 298-301; 305-308; 492-495; 551-554) were obtained for each protein sequence of HA isolated since 1998 until 2011. With the Exception of vaccine strain in 1998 which did not have glycosylation site at position(551-554).

More amino acid substitutions and variations were also observed for NA protein sequences. Nine glycosylation sites for the dominant viral strains from 1998 to 2006 were similar to each other but viral strains isolated in 2011 revealed five new modifications at N-glycosylation site NSSK, NGTI, NRSG, NSSE, NVTE at positions 44-47, 69-72, 402-405, 44-77, 61-64 in A/Ck/Ir/N101/2011, A/CK/Ir/191/2011, A/Ck/Ir/187/2011, A/Ck/Ir/186/2011, A/Ck/Ir/198/2011 Respectively and one new glycosylation site NSSY at position 313-316 in A/Ck/Ir/189/2011.

The hemadsorbing (HB) site

Amino acids of HB site in the NA stalk are at three locations, the first position is 366-373, the second one is 399-404 and the third one is 431-433. HB sites at position 366-373 were IKKDSRAG except for A/ck/Iran/b111/2004 and A/ck/Iran/68/2006 isolates which was IKQDSRAG and A/ck/Iran/SH2/2007 isolate which was IKKDSREG. A/ck/Iran/RZ36/2008 and A/ck/Iran/RZ71/2009 were IRKDSRAG. HB site at position 399-404 was DSDNLS except for A/ck/Iran/RZ36/2008 and A/ck/Iran/RZ71/2009 which was DSDNWS and all the strains isolated in this study which was DSDNRS. The other situation contained single type PQE at position 431-433 (Table 2).

Phylogenetic analysis

Evolutionary relationship of HA and NA nucleotides sequence were determined by comparing these Iranian isolates and selected H9N2 viruses isolated in several other countries with the established Eurasian H9N2 lineages: namely, the G1-lineage, the Y280-lineage and the Korean-lineage represented by prototype viruses A/Quail/Hong Kong/G1/97, A/Duck/Hong Kong/Y280/97 and A/Chicken/Korea/38349-p96323/96 respectively, and with viruses identified in the Far East as emerging H9N2 lineages(Xu et al., 2007a).The strain of current vaccine was also included in this analysis. Phylogenetic analysis of the HA gene indicates that all Iranian isolates belong to the A/Qail/HongKong/G1/97-like virus sublineage, (Figure 1). The strains which were

isolated in this study had close relationship with other Iranian strains which were dominant during 2009-2011 suggesting that they are from common source. Moreover the strong relationship was observed with H9N2 viruses from Pakistan, Saudi Arabia, Egypt and Israel respectively. In turn, the Iranian isolates (this isolates and the others) can be divided in to three subgroups: The first of these are viruses isolated since 1998 to 2005. The Iranian strains isolated during 2004-2008 fall in to the second subgroup. Those strains isolated during 2009 to 2011 and new strains in this study fall into the third subgroup (Figure1). Therefore these three subgroups are present in the country since 1998. None of the sequences clustered within the Y280 and Korean-like lineages.

Phylogenetic analysis of N2 gene showed that all the NA genes of the Iranian H9N2 viruses fall into G1-like lineage (Fig.2). These findings further confirm the Asian origins of the Iranian viruses. In turn, this group is divided into subgroups: the first group includes viruses isolated from 1998 to 2007, the second subgroup includes strains isolated during 2008 -2011.

Epitope analysis of HA and NA for antigenic variations

Results from CTLPred epitope prediction showed that HA protein sequences isolated from Iran had some variations. Epitope GNCPKYVRV which was observed in position 312 of the vaccine strain (A/Ck/Ir/av1221/98), was absent in current strains except for Ck/Ir/191/2011 that was GTCPKYISL. Another epitope (IGLRNVPAK) at position

327-336 that was seen in Ck/Ir/189/2011 had not been found in previously reported strains in Iran. Furthermore, viruses isolated since 2004 to 2011 and viruses isolated in this study revealed SFYRNMRWL epitope at position 154-163 that was absent in vaccine strain(Table 5).

Epitopes for NA showed lots of variations in a yearly manner. More observations revealed that GANINFMSI and HRTLLMNEL epitopes at position 461-469 and 155-163, were present in 1998 and vaccine strains, respectively. But were absent in viruses isolated in this study. Instead SCYPRYPEV, SCHDGRAWL and SELGVPFHL epitopes at positions 279-287, 182-190 and 161-169 which are present in strains in 2011 were not seen in 1998 and vaccine strain. Another change was that DYNINSSYL epitope was only present in Ck/Ir/189/2011(Table 6).

Table 1 : Receptor binding site and cleavage site of H9N2 viruses

Viruses	98 (106)	153 (161)	155 (163)	183 (191)	190 (198)	194 (202)	195 (203)	226 (234)	cleavage site 335-339
A/Ck/HK/G1/97	G	W	T	H	E	L	Y	L	RSSR
A/Ir/av1221/98	-	-	-	-	A	-	-	-	RSSR
A/Ck/Ir/TH77/98	-	-	-	-	A	-	-	-	RSSR

A/Ck/Ir/661/98	-	-	-	-	A	-	-	-	RSSR
A/Ck/Ir/584/20	-	-	-	-	A	-	-	Q	RSSR -
A/Ck/Ir/TH81/02	-	-	-	-	A	-	-	Q	RSSR
A/Ck/Ir/320/03	-	-	-	-	A	-	-	-	RSSR
A/Ck/Ir/B76/04	-	-	-	-	A	-	-	-	RSSR
A/Ck/Ir/152/04	-	-	-	-	A	-	-	-	RSSR
A/Ck/Ir/B102/05	-	-	-	-	A	-	-	-	RSSR
A/Ck/Ir/119/05	-	-	-	-	A	-	-	Q	RSSR -
A/Ck/Ir/20/06	-	-	-	-	A	-	-	-	RSSR
A/Ck/Ir/SH2/07	-	-	-	-	A	-	-	-	RSSR
A/Ck/Ir/R36/08	-	-	-	-	A	-	-	-	RSSR
A/Ck/Ir/SS2/08	-	-	-	-	A	-	-	-	RSSR
A/Ck/Ir/EBVG/10	-	-	-	-	T	-	-	-	KSSR
A/Ck/Ir/ 186/11	-	-	-	-	A	-	-	-	KSSR
A/Ck/Ir/ 187/11	-	-	-	-	A	-	-	-	KSSR
A/Ck/Ir/ 189/11	-	-	-	-	A	-	-	-	KSSR
A/Ck/Ir/191/11	-	-	-	-	A	-	-	-	KSSR

Table 2 :Amino acid substitution in the hemadsorbing sites and deletion in stalk

		Site HB		
Viruses	Deletion in stalk	366-373	399-404	431-433
A/Ck/Iran/av1221/98	-	IKKDSRAG	DSDNLS	PQE
A/ Ck/Iran/661/1998	-	IKKDSRAG	DSDNLS	PQE
A/Ck/Iran/11T/99	-	IKKDSRAG	DSDNLS	PQE
A/Ck/Iran/284/2002	-	IKKDSRAG	DSDNLS	PQE
A/Ck/Iran/B111/2004	-	IK**Q**DSRAG	DSDNLS	PQE
A/Ck/Iran/68/2006	-	IK**Q**DSRAG	DSDNLS	PQE
A/ Ck/Iran/SH2/2007	-	IKKDSR**E**G	DSDNLS	PQE
A/ Ck/Iran/RZ36/2008	-	I**R**KDSRAG	DSDN**W**S	PQE
A/Ck/Iran/RZ53/2008	-	I**R**KDSR**E**G	DSDNLS	PQE
A/Ck/Iran/RZ71/2009	-	I**R**KDSRAG	DSDN**W**S	PQE
A/Ck/Iran/N101/2011	-	IKKDSRAG	DSDN**R**S	PQE
A/Ck/Iran/186/2011	-	IKKDSRAG	DSDN**R**S	PQE
A/Ck/Iran/187/2011	-	IKKDSRAG	DSDN**R**S	PQE

A/Ck/Iran/189/2011	-	IKKDSRAG	DSDN**R**S	PQE
A/Ck/Iran/191/2011	-	IKKDSRAG	DSDN**R**S	PQE
A/QA/HK/G1/97	38-39	IKKDSRSG	DSDIRS	PQE
A/HK/1074/99	38-39	IKKDSRSG	DSDNWS	PQE
A/DK/HK/Y280/97 H9N2	63-65	IKEDSRSG	DSDNWS	PQE

Table 3.comparison of predict N-glycosylation sites (PGS)within aminoacid sequences of HA proteins isolated between 1998-2011(H9N2).

Strain	position						
	29-32	105-108	141-144	298-301	305-308	492-495	551-554
A/Ir/av1221/98	NSTE	NGTC	NVTY	NSTL	NISK	NGTY	-
A/Ck/Ir/661/1998	NSTE	NGTC	NVTY	NSTL	NISK	NGTY	-
A/Ck/Ir/TH77/1998	NSTE	NGTC	NVTY	NSTL	NISK	NGTY	NGSC
A/Ck/Ir/TH81/2002	NSTE	NGTC	NVTY	NSTL	NISK	NGTY	NGSC
A/Ck/Ir/B76/2004	NSTE	NGTC	NVTY	NSTL	NISK	NGTY	NGSC
A/Ck/Ir/TH81/2002	NSTE	NGTC	NVTY	NSTL	NISK	NGTY	NGSC
A/Ck/Ir/B76/2004	NSTE	NGTC	NVTY	NSTL	NISK	NGTY	NGSC
A/Ck/Ir/TH186/2007	NSTE	NGTC	NVTY	NSTL	NISK	NGTY	NGSC
A/Ck/Ir/RZ53/2008	NSTE	NGTC	NVTY	NSTL	NISK	NGTY	NGSC
A/Ck/Ir/RZ36/2008	NSTE	NGTC	NVTY	NSTL	NISK	NGTY	NGSC
A/Ck/Ir/EBGV/2010	NSTE	NGTC	NVTY	NSTL	NISK	NGTY	NGSC
A/Ck/Ir/N101/2011	NSTE	NGTC	NVTY	NSTL	NISK	NGTY	NGSC

A/Ck/Ir/186/2011	NSTE	NGTC	NVTY	NSTL	NISK	NGTY	NGSC
A/Ck/Ir/187/2011	NSTE	NGTC	NVTY	NSTL	NISK	NGTY	NGSC
A/Ck/Ir/189/2011	NSTE	NGTC	NVTY	NSTL	NISK	NGTY	NGSC
A/Ck/Ir/191/2011	NSTE	NGTC	NVTY	NSTL	NISK	NGTY	NGSC

Table 4. comparison of predict N-glycosylation sites (PGS)within aminoacid sequences of NA proteins isolated between 1998-2011(H9N2).

	position								
Viruses	44-47	61-64	69-72	70-73	86-89	146-149	200-203	234-237	313-316 402-405
A/Ck/Ir/av1221/98	NPSN	NITE	NNTT	NTTI	NWSK	NGTT	NATA	NGTC	- NLSG
A/Ck/Ir/661/1998	NPSN	NITE	NNTT	NTTI	NWSK	NGTT	NATA	NGTC	- NLSG
A/Ck/Ir/ZMT/101/98	NPSN	NITE	NNTT	NTTI	NWSK	NGTT	NATA	NGTC	- NLSG
A/Ck/Iran/11T/99	NPSN	NITE	NNTT	NTTI	NWSK	NGTT	NATA	NGTC	- NLSG
A/Ck/Iran/284/2002	NPSN	NITE	NNTT	NTTI	NWSK	NGTT	NATA	NGTC	- NLSG
A/Ck/Iran/B111/2004	NPSN	NITE	NNTT	NTTI	NWSK	NGTT	NATA	NGTC	- NLSG
A/Ck/Iran/68/2006	NPSN	NITE	NNTT	NTTI	NWSK	NGTT	NATA	NGTC	- NLSG
A/Ck/Iran/RZ53/2008	-	N**R**TE	NNTT	NTTI	NWSK	NGTT	NATA	NGTC	- NLSG
A/Ck/Iran/RZ36/2008	-	NITE	N**G**T**I**	-	NWSK	NGTT	NATA	NGTC	- N**W**SG

A/Ck/Iran/RZ71/2009	-	NITE	N**G**T**I**	-	NWSK	NGT	NATA	NGTC	-	N**W**SG
A/Ck/Iran/N101/2011	N**S**S**K**	NITE	N**G**T**I**	-	NWSK	NGTT	NATA	NGTC	-	N**R**SG
A/Ck/Ir/191/2011	N**S**S**K**	NITE	N**G**T**I**	-	NWSK	NGTT	NATA	NGTC	-	N**R**SG
A/Ck/Ir/187/2011	N**S**S**E**	NITE	N**G**T**I**	-	NWSK	NGTT	NATA	NGTC	-	N**R**SG
A/Ck/Ir/186/2011	N**S**S**K**	N**V**TE	N**G**T**I**	-	NWSK	NGTT	NATA	NGTC	-	N**R**SG
A/Ck/Ir/189/2011	N**S**S**K**	NITE	N**G**T**I**	-	NWSK	NGTT	NATA	NGTC	**NSSY**	N**R**SG

	position							
Viruses	431	108	134	154	312	324	327	493

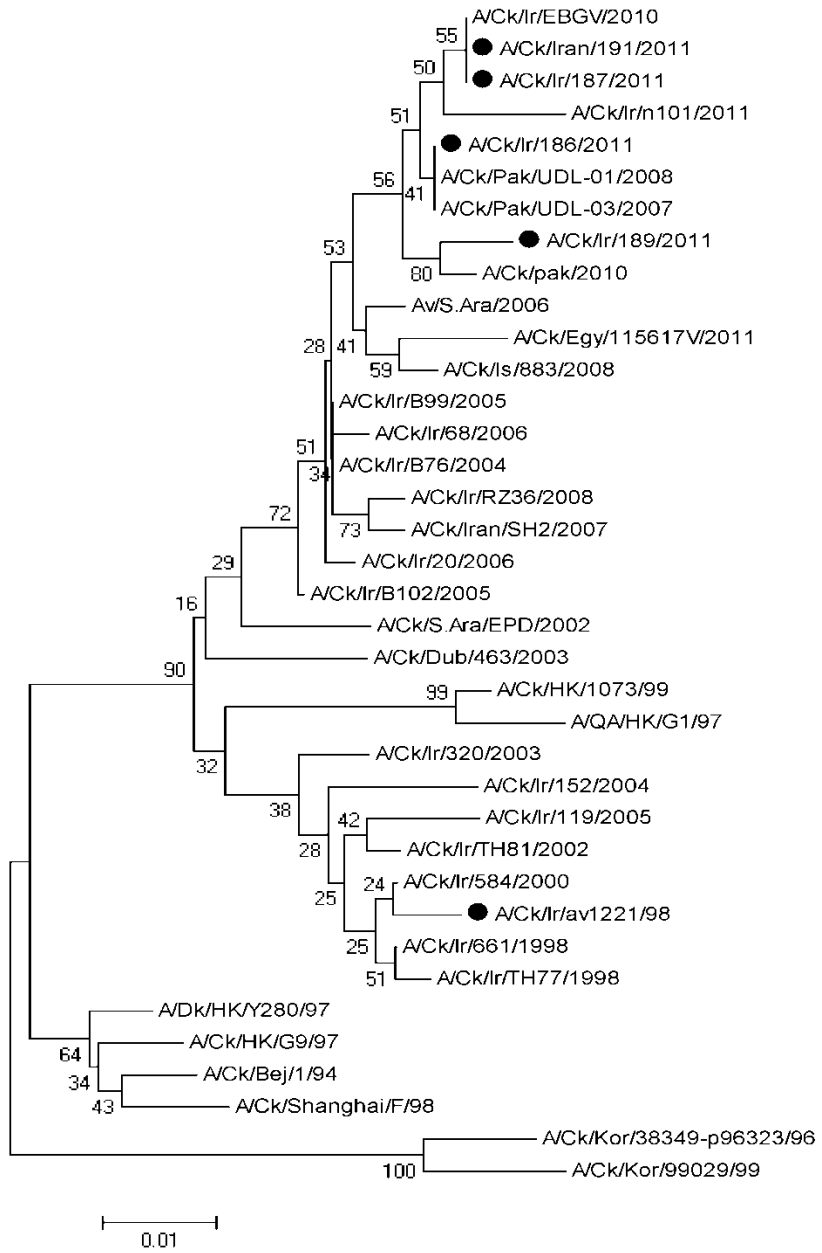
Table 5. Comparison of antigenic epitope in the amino acid sequences of HA protein (H9N2) from 1998 to 2011.

A/Ck/Ir/av1221/98	AYNAELLVL	CYPGNVENL	IFPDTIWNV	-	GNCPKYVRV	KLAVGLRNV	-	-
A/Ck/Ir/TH7798	AYNAELLVL	CYPGNVENL	IFPDTIWNV	-	GNCPKYVRV	KLAVGMRNV	-	-
A/Ck/Ir/661/98	AYNAELLVL	CYPGNVENL	IFPDTIWNV	-	GNCPKYVRV	KLAVGLRNV	-	-
A/Ck/Ir/TH81/2002	AYNAELLVL	-	IFPDTIWNV	-	GNCPKYVRV	KLAVGLRNV	-	GTYDRRKYK
A/Ck/Ir/B76/2004	AYNAELLVL	CYPGNVENL	IFPDTIWNV	SFYRNMRWL	-	KLAVGLRNV	-	-
A/Ck/Ir/TH186/2007	AYNAELLVL	CYPGNVENL	IFPDTIWNV	SFYRNMRWL	-	KLAVGLRNV	-	-
A/ Ck/Ir/SH2/2007	AYNAELLVL	CYPGNVENL	IFPDTIWNV	SFYRNMRWL	-	KLAVGLRNV	-	-
A/Ck/Ir/RZ53/2008	AYNAELLVL	CYPGNVENL	IFPDTIWNV	SFYRNMRWL	-	KLAVGLRNV	-	-
A/Ck/Ir/RZ36/2008	AYNAELLVL	CYPGNVENL	IFPDTIWNV	SFYRNMRWL	-	KLAVGLRNV	-	-
A/Ck/Ir/EBGV/2010	AYNAELLVL	CYPGNVENL	IFPDTIWNV	SFYRNMRWL	-	KLAVGLRNV	-	-
A/Ck/Ir/186/2011	AYNAELLVL	CYPGNVENL	IFPDTIWNV	SFYRNMRWL	-	KLAVGLRNV	-	-
A/Ck/Ir/191/2011	AYNAELLVL	CYPGNVENL	IFPDTIWNV	SFYRNMRWL	GTCPKYISL	-	-	-
A/Ck/Ir/187/2011	AYNAELLVL	CYPGNVENL	IFPDTIWNV	SFYRNMRWL	-	KLAVGLRNV	-	-
A/Ck/Ir/1892011	AYNAELLVL	CYPGNVENL	-	SFYRNMRWL	-	KLAVGLRNV	IGLRNVPAK	-

Table 6. Comparison of antigenic epitope in the amino acid sequences of NA protein (H9N2) from 1998 to 2011.

	position									
Viruses	**100**	**297**	**248**	**461**	**155**	**279**	**299**	**182**	**161**	**309**
A/Ck/Ir/av1221/98	FSKDNSIRL	GSNRPVLYI	GRADTRILF	GANINFMSI	HRTLLMNEL	-	-	-	-	-
A/Ck/Ir/661/98	FSKDNSIRL	GSNRPVLYI	GRADTRILF	GANINFMSI	HRTLLMNEL	-	-	-	-	-
A/Ck/Ir/ZMT/101/98	FSKDNSIRL	GSNRPVLYI	GRADTRILF	GANINFMSI	HRTLLMNEL	-	-	-	-	-
A/Ck/Ir/11T/99	FSKDNSIRL	GSNRPVLYI	GRADTRILF	GANINFMSI	HRTLLMNEL	-	-	-	-	-
A/Ck/Ir/284/2002	FSKDNSIRL	GSNRPVLYI	GRADTRILF	GANINFMSI	HRTLLMNEL	-	-	-	-	-
A/Ck/Ir/B112004	FSKDNSIRL	GSNRPVLYI	GRADTRILF	-	HRTLLMNEL	-	-	-	-	-
A/Ck/Ir/68/2006	FSKDNSIRL	GSNRPVLYI	GRADTRILF	-	HRTLLMNEL	SCYPRYPEV	-	-	-	-
A/Ck/Ir/SH2/2007	FSKDNSIRL	GSNRPVLYI	-	GANINFMSI	HRTLLMNEL	SCYPRYPEV	-	-	-	-
A/Ck/Ir/RZ36/2008	FSKDNSIRL	GSNRPVLYI	-	GANINFMSI	-	SCYPRYPEV	NRPVLYINM			
A/Ck/Ir/RZ53/2008	FSKDNSIRL	GSNRPVLYI	-	GANINFMSI	HRTLLMNE L	SCYPRYPEV	-	-	-	-
A/Ck/Ir/RZ71/2009	FSKDNSIRL	GSNRPVLYI	-	GANINFMSI	-	SCYPRYPEV	NRPVLYINM	-	-	-
A/Ck/Ir/N1012011	FSKDNSIRL	GSNRPVLYI	GRADTRILF	-	-	SCYPRYPEV	-	SCHDGRAWL	-	-
A/Ck/Ir/191/2011	FSKDNSIRL	GSNRPVLYI	-	-	-	SCYPRYPEV	-	SCHDGRAWL	SELGVPFHL	-

A/Ck/Ir/186/2011	FSKDNSIRL	GSNRPVLYI	-	-	-	SCYPRYPEV	-	SCHDGRAWL	SELGVPFHL	-	
A/Ck/Ir/189/2011	FSKDNSIRL	GSNRPVLYI	GRADTRILF	-	-	-	-	SCHDGRAWL	-	DYNINSSYL	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A/Ck/Ir/186/2011
A/Ck/Pak/UDL-01/2008
A/Ck/Pak/UDL-03/2007
A/Ck/Ir/189/2011
A/Ck/pak/2010
Av/S.Ara/2006
A/Ck/Egy/115617V/2011
A/Ck/Is/883/2008
A/Ck/Ir/B99/2005
A/Ck/Ir/68/2006
A/Ck/Ir/B76/2004
A/Ck/Ir/RZ36/2008
A/Ck/Iran/SH2/2007
A/Ck/Ir/20/2006
A/Ck/Ir/B102/2005
A/Ck/S.Ara/EPD/2002
A/Ck/Dub/463/2003
A/Ck/HK/1073/99
A/QA/HK/G1/97
A/Ck/Ir/320/2003
A/Ck/Ir/152/2004
A/Ck/Ir/119/2005
A/Ck/Ir/TH81/2002
A/Ck/Ir/584/2000
A/Ck/Ir/av1221/98
A/Ck/Ir/661/1998
A/Ck/Ir/TH77/1998
A/Dk/HK/Y280/97
A/Ck/HK/G9/97
A/Ck/Bej/1/94
A/Ck/Shanghai/F/98
A/Ck/Kor/38349-p96323/96
A/Ck/Kor/99029/99
55
50
51
56
41
53
80
28
41
59
51
34
72
73
29
16
90
99
32
38
42
28
25
24
25
51
64
34
43
100
0.01

Figure 1:Phylogenetic relationship of HA gene from H9N2 virus isolated in Iran during 1998-2011 and other reperesentative AIV_S of this subtype. The strains presented in this study are indicated as character ●

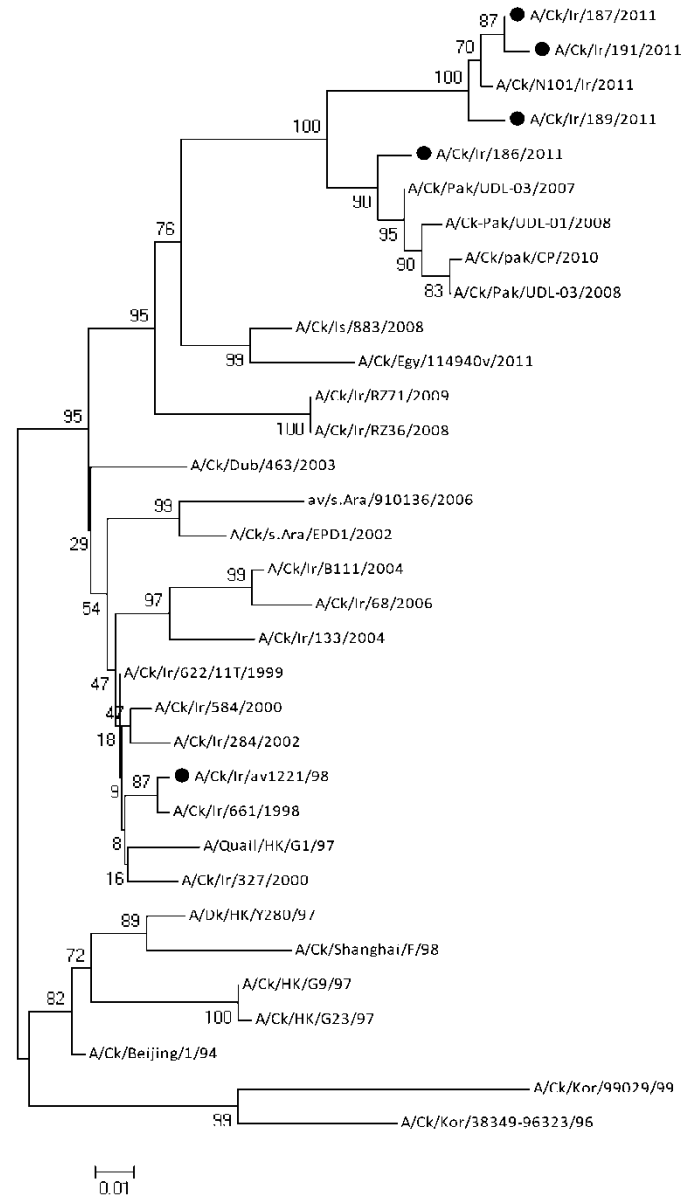
A/Ck/Ir/187/2011
A/Ck/Ir/191/2011
A/Ck/N101/Ir/2011
A/Ck/Ir/189/2011
A/Ck/Ir/186/2011
A/Ck/Pak/UDL-03/2007
A/Ck-Pak/UDL-01/2008
A/Ck/pak/CP/2010
A/Ck/Pak/UDL-03/2008
A/Ck/Is/883/2008
A/Ck/Egy/114940v/2011
A/Ck/Ir/R771/2009
A/Ck/Ir/RZ36/2008
A/Ck/Dub/463/2003
av/s.Ara/910136/2006
A/Ck/s.Ara/EPD1/2002
A/Ck/Ir/B111/2004
A/Ck/Ir/68/2006
A/Ck/Ir/133/2004
A/Ck/Ir/G22/11T/1999
A/Ck/Ir/584/2000
A/Ck/Ir/284/2002
A/Ck/Ir/av1221/98
A/Ck/Ir/661/1998
A/Quail/HK/G1/97
A/Ck/Ir/327/2000
A/Dk/HK/Y280/97
A/Ck/Shanghai/F/98
A/Ck/HK/G9/97
A/Ck/HK/G23/97
A/Ck/Beijing/1/94
A/Ck/Kor/99029/99
A/Ck/Kor/38349-96323/96
0.01

Figure 2:Phylogenetic relationship of NA gene from H9N2 virus isolated in Iran during 1998-2011 and other reperesentative AIV_S of this subtype.The strains presented in this study are indicated as character ●

Discution

As one of the major surface glycoproteins of Influenza A viruses, HA actually specifies several important functions. The globular tip of the HA protein contains a receptor for binding to the host cell surface (Perdue. 2008). Antibodies made by the infected host and directed at the globular tip effectively attack the HA and prevent binding and entry. However, this globular head also changes due to mutations rapidly enough to produce novel structural components that are no longer recognized by host cell antibodies. This “antigenic drift” in effect gives the virus an “escape” mechanism from antibodies to once again allow efficient binding and entry to the cell. This feature of the HA is the reason that twice yearly the World Health Organization convenes experts to evaluate antigenic and genomic changes and recommend modifications to human vaccine strains according to the changes in the HA in the seasonal influenza human strains(Perdue. 2008). Sequence and phylogenetic analysis have the potential to be effective approaches towards vaccine development(Xu et al., 2007b; Park et al., 2011).Therefore, the present study was performed to verify the extent of antigenic

variation in HA and NA genes and deduced surface proteins sequences of the H9N2 avian virus isolated in Iran.

Analysis of the HA cleavage site showed that strains isolated in 2011 had motife (KSSR/GLF) that was different from those previously reported in Iran. From 1998 to 2008, all H9N2 viruses retained a conserved amino acid pattern at the cleavage site; RSSR/GLF(Vasfi Marandi and Bozorgmehri-Fard. 2002; Karimi et al., 2004; Kianizadeh et al., 2006; Soltanialvar et al., 2010). The motif (KSSR/GLF)might indicate the LPAI nature of H9N2 strains,however the significance of this mutation on viral stability or increased pathogenicity requires further studies, as the cleavage site is considered an indicator of pathogenicity(Senne et al., 1996; Hulse et al., 2004).
It has been well documented that the receptor binding site motif of HA is critical for cellular receptor specificity and determining virus host range(Ha et al., 2001; Gambaryan et al., 2002). The presence of glutamine (Q) at position 234 (H3 numbering: 226) is the typical avian virus signature, and it has been reported that presence of this amino acid results in affinity for binding to 2,3-linked sialic acid (avian receptors), whereas in the case of leucine (L) at the same position, there is a affinity for 2,6-linked sialic acid (human receptors) and potential cause of reported human infections (Lin et al., 2000) Therefore, we could predict that the Iranian isolates in this study have affinity for binding to human like receptor which are similar to it of other strains isolated in Iran(Soltanialvar et al., 2010; Bozorgi et al., 2012)

Glycosylation plays an important role in the viral life cycle. Glycans near the antigenic epitopes interfere with antibody recognition to escape immune pressure from hosts, and those near HA0 cleavage site protect their infectivity(Skehel et al., 1984; Deshpande et al., 1987; Igarashia et al., 2008) Gain or loss of glycosylation sites near the receptor-binding domain can also affect viral receptor recognition(Das et al., 2010; Chen et al., 2011) . Deshpande demonstrated that the only difference in glycosylation between HA1 (virulent H5N2) and HA1 (avirulent H5N2) is the absence of an N-linked oligosaccharide at Asn residue 11 in HA1 (virulent H5N2). This result showed that a site-specific glycosylation can regulate the processing of HA and thus the virulence of a virus(Deshpande et al., 1987).

In this study glycosylation motifs NGTC, NISK and NGTY were located near antigenic epitopes CYPGNVENL, GTCPKYISL, GTYDRRKYK at positions 108, 312 and 493 in A/ck/Iran/187, A/ck/Iran/191 and A/ck/Iran/TH81/02, respectivly. Furthermore, glycosylation of the NA gene with motif NSSY at position 313-316 was located near DYNINSSYL epitope at position 309 in A/ck/Iran/189 strain. These variations may interfere with antibody recognition and help the virus to escape from immune responses. Computational and experimental studies of currently reported HA and NA segments from Influenza A viruses, will be useful for determining antigenic variation and its influence on virulence of these strains.

Sequence analysis revealed that mutation of the NA hemadsorption site in H9N2 viruses resulted from a positive selective pressure to change suggesting that a loss of

the site increased virus fitness in land-based poultry (Matrosovich et al., 2001) Hemadsorption site serves to enhance the catalytic efficiency of NA and, in addition to changes in the receptor-binding specificity of the hemagglutinin, alterations of the NA are needed for the emergence of pandemic influenza viruses (Uhlendorff et al., 2009).The HB site in the NA of Asian H9N2 viruses is believed to be under positive selection pressure for mutations, which results in compatible combinations of HA and NA (Matrosovich et al., 2001).

It is remarkable that an inactive H9N2 vaccine which has extensively been used to control avian influenza is responsible for the mutation observed in HB site in the NA genes of Iranian H9N2 viruses, resulting from pressure of vaccination program on immune system. In addition, the virus circulation in the region might has also increased these modification. Despite all these changes in viruses of Iranian isolates they still have a relationship with the G1 sublineage.

The sequence and length of the stalk region is known to vary among and within NA subtypes. Shortening of the NA stalk by deletion of the amino acid is characteristic of the highly pathogenic H5 and H7 influenza viruses (Matrosovich M Fau - Zhou et al., 1999). However, it is not known whether deletion in the NA of H9N2 virus is correlated with pathogenicity in chickens. The NA stalk regions in these four viruses, like Iranian sequence available in GenBank had no deletions in the stalk (Table 2)

Phylogenetic analysis revealed that Iranian isolates had fallen within distinct clusters for several years. It seems as if these viruses are being faced with two phenomena: accumulation of mutations and reassortment in the G1-like sublineage(Vatandour et al., 2011). Evolution relationship showed that current strains isolated in Iran in 2011 are closely related to strains isolated in Pakistan where has common boundaries with Iran. On the other hand they are also similar to viruses isolated in Egypt in 2011 and in Israel in 2008 as well as Saudi Arabia in 2006 where do not have any common boundaries with Iran.It seems viruses isolated from Iran,Egypt,Israel and Saudi Arabia have originated from common source in spite of being geographical distances.

It was observed based on phylogenetic analysis that the vaccine strain and the 1998's strain from Iran appear in the same subgroup and shared a homology of 99.2 in both NA and HA amino acids. Viruses isolated in 2011 fall into another subgroup and compare with vaccine strain displayed amino acid homology of HA and NA ranging from 90.4% to 91.1% and 90.7% to 91.4%, respectively. Results revealed that these strains had non negligible genetic differences with the vaccine strains. The potential for differences in antigenic variations is high, especially when compared with sublineages of H9N2 viruses(Xu et al., 2007a).It has been demonstrated antigenic diversities in H9N2 viruses using monoclonal antibodies correspond with phylogenetic relationships(Xu et al., 2007b). Their findings also correspond with our results where H9N2 viruses showed sequence variations and antigenic diversities based on evolution. Three and seven differences in antigenic epitopes observed in HA and NA genes of

current isolates and vaccine strain respectivly(Table 5,6) in addition to five glycosilation motifs and one new glycosylation site at current strains(Table 4,5) might trigger antigenic diversities. Therefore, continues evaluation of important gene induced immunity and vaccine efficiency in Iran would be crucial.

Conclusion: Findings from the study support the genetic instability of influenza A (H9N2) viruses.. The genetic disparity between the current prevalent strains and vaccine strain may result to decreased protection after vaccination. Thus in the countries where a control policy is established these divergenisities should be considered. Our study would laid new foundation for genetic evaluation of H9N2 viruses compared with vaccine strain and necessitated yearly surveillance in Iran.

Abbreviations:

HA:Hemagglutinin ,NA:Neuraminidase,PGS:Potential glycosylationsite,HB:Hemadsobing site,AIV:Avian influenza virus,nHPAI:non-highly pathogenic avian influenza,LPAI:Low pathogenic avian influenza,OIE:office international des Epizootics,RT-PCR:reverse transcription polymerase chain reaction,A:influenza A, Ck:chicken,Ir:Iran,Pak:Pakistan,Egy:Egypt,Av:Avian,Ara:Arabia,Dub:Dubai,HK:HongKong,QA:Quail,Kor:Korea,DK:Duck

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