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Phylogenetic analysis of the hemagglutinin gene of recent H9N2 avian influenza viruses isolated from broiler flocks in Iran

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ABSTRACT

In the present study, the genetic variations were examined of hemagglutinin genes of 4 recent H9N2 subtype avian influenza viruses isolated in the Tehran province of Iran. These hemagglutinin genes were amplified and sequenced in order to compare these viruses with the previous isolates from Iran and some other countries from all over the world. The full length hemagglutinin genes of 112 H9N2 avian influenza strains isolated from chickens, and also migratory birds from all over the world, including 68 strains isolated in Iran during 1998-2012, were genetically analyzed. The amino acids in the hemagglutinin cleavage site of the all 4 recent isolates possessed a PAKSSR/GL motif, which is a different motif when compared with the predominant PARSSR/GL motif in the hemagglutinin of the Iranian H9N2 strains isolated before 2010. All 4 recent isolates possessed histidine, alanin, leucine and isoleucine at positions 183, 190, 226 and 227 respectively, which are the key residues in receptor-binding sites. Genetic and phylogenetic analysis of the hemagglutinin genes showed that recent H9N2 strains isolated from chickens in Iran during 2010-2012, formed a distinct subgroup of the previous strains. Recent changes in the H9N2 viruses may be the result of the widespread circulation of these viruses in recent years. This may be due to the lack of adequate surveillance and control programs, such as vaccination and quarantine. Hence, it is highly recommended that continuous surveillance programs and genetic analysis of ongoing changes to H9N2 should be considered.

Key words: avian influenza virus, H9N2, hemagglutinin, phylogenetic analysis, Iran

Introduction

All influenza viruses are currently members of the family *Orthomyxoviridae*. Although influenza viruses are divided into three different types (A, B, and C), on the basis of

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antigenic differences among their nucleopcapsid (NP) and matrix (M) proteins, all avian influenza (AI) viruses are classified as type A influenza virus (SWAYNE and HALVORSON, 2008). Avian influenza (AI) surveillance studies first started in the 1970s, and have led to the identification of 16 hemagglutinin and nine neuraminidase subtypes, on the basis of the antigenic relationships of the hemagglutinin (HA) and neuraminidase (NA) surface glycoproteins of AI viruses (LUPIANI and REDDY, 2009). Moreover, neutralizing antibodies, produced against the rod-shaped trimers of the HA surface glycoproteins of the virus envelope, play an important role in a protective immune response, but such protections are only subtype specific (SUAREZ and SCHULTZ, 2000).

Numerous H9N2 subtype AI outbreaks have been reported over the last decade in Germany, Italy, Ireland, South Africa, the United States of America (USA), Korea, China, some countries of South East Asia and the Middle East, especially Pakistan and also Iran. In fact, H9N2 AI is now considered enzootic throughout Asia (ALEXANDER, 2007). Although H9N2 subtype AI viruses from poultry farms in Iran have been characterized as a low pathogenic AI (LPAI) in experimental conditions (VASFI MARANDI and BOZORGMEHRI FARD, 2002) and nearly all broiler flocks in Iran received an inactivated oil emulsion LPAI vaccine, which often contained a strain from the earliest outbreaks in Iran in 1998, high mortality up to 60 % in field conditions has been reported from Iran, which causes severe financial losses (NILI and ASASI, 2003; NILI and ASASI, 2002).

Although there has been little evidence of human-to-human transmission of H9N2 viruses, and these viruses have been sporadically introduced into humans (GUO et al., 2001; GUO et al., 1999; LIN et al., 2000; PEIRIS et al., 1999; UYEKI et al., 2002), serological studies have shown the presence of antibodies against the H9 subtype in the human population of Iran (MOOSAKHANI et al., 2010). In addition, the low pathogenic nature of H9N2 viruses for birds can provide increased opportunities for reassortment that can lead to an increase in the affinity of H9N2 viruses for human influenza receptors, and they may acquire the ability for human-to-human transmission (HA et al., 2001; HA et al., 2002; NAEEM and HUSSAIN, 1995). Therefore, it is important to consider H9N2 viruses as an important candidate for a human pandemic virus, and conduct more studies on it (LUPIANI and REDDY, 2009).

The objective of this study was to examine the genetic variations of hemagglutinin genes of 4 recent H9N2 subtype AI viruses, isolated in the Tehran province of Iran, in order to compare these viruses to the previous isolates, and also to examine the molecular epidemiology of H9N2 in the poultry population of this country.

Materials and methods

Viruses. The H9N2 strains were isolated from epithelial scrapings of the tracheal mucosa of poultry deaths referred to the laboratory from different poultry farms in the

Tehran province of Iran during January and March, 2012. All samples were obtained from 30-50-day-old broiler farms that had not been vaccinated against AI viruses. All the farms referred the carcasses with a history of a high mortality rate (more than 30 %) and various combinations of other agents. Almost all the carcasses showed the signs of upper respiratory tract involvement, such as: mucous-to-mucopurulent nasal discharge, serous-to-caseous tracheal exudates and fibrinopurulent bronchopneumonia. The study addressed 4 H9N2 viruses isolated from chicken flocks. The isolates were propagated in 10-day-old specific-pathogen free (SPF) embryonating chicken eggs (Valo, Lohman, Germany) via the chorioallantoic sac (CAS). The allantoic fluids collected from embryonating chicken eggs, which were positive by the hemagglutination test, were used for virus identification. Hemagglutination inhibition (HI) and neuraminidase inhibition (NI) tests were used for serological identification of the virus subtypes of the AI virus isolates, with monospecific polyclonal chicken antisera (CVL, Weybridge, Surrey, UK) (BEARD, 1989a; BEARD, 1989b). Allantoic fluids were used as stock viruses for further studies.

RT-PCR and sequence analysis. The viral RNA was extracted from stock viruses in the infected allantoic fluids using a High Pure Viral RNA Kit (Roche, Germany) according to the manufacturer's instructions. Purified genomic RNA was used to generate cDNA clones by reverse transcription polymerase chain reaction (RT-PCR) according to the standard procedure. The RT-PCR was carried out using the Titan One Tube RT-PCR System (Roche, Germany). The PCR Amplification of the HA precursor coding sequence (HA1 and HA2) was carried out using two sets of primers: the first set AIha1F and AIha1R (1111bp), and the second set AIha2F and AIha2R (950bp) (LIU et al., 2003; MOOSAKHANI et al., 2010). The primer sets were used for the RT-PCR reaction and also for the subsequent sequence analysis (the sequences of the primers are available on request).

The RT-PCR reactions were subjected to a reverse transcription of the viral RNA at 45 °C for 45 min, initial denaturation at 94 °C for 2 min and 35 cycles consisting of 94 °C for 30 sec (denaturation), 57 °C for 45 sec (annealing), and 68 °C for 2 min (extension). The final extension step was extended to 10 min at 68 °C.

The PCR products were subjected to electrophoresis in a 0.9 % (w/v) agarose gel, and then the DNA fragments of the expected length of 1111 base pairs (bp) and 950 bp were extracted and purified using a High Pure PCR Product Purification kit (Roche, Germany). The purified DNA fragments were sequenced at GATC Biotech Ltd., Germany, using Sanger sequencing on ABI 3730 DNA sequencers.

BioEdit software was used to assemble and translate the nucleotide sequences into protein sequences. Percent identity and divergence among nucleotide sequences were computed from 'Sequence Distances' in DNASTAR Lasergene MegAlign. Phylogenetic analysis was carried out for 1683 nucleotides, which were used in the clustal W alignment

method, Tamura-Nei substitution model and 1000-replicate Bootstrap test of phylogeny in MEGA5. A total of 112 nucleotide sequences of HA genes were selected for genetic analysis, including all the HA gene nucleotide sequences from Iran (68 nucleotide sequences) that were available up to August 12, 2012 in the GenBank sequence database provided by the National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov/genbank). Then a total of 57 nucleotide sequences, in which 28 out of 68 Iranian isolate HA sequences could be seen among them, were chosen for phylogenetic and other analyses that are presented in this study.

Nucleotide sequences accession numbers. The nucleotide sequences for all H9N2 influenza viruses isolated in this study are available in GenBank under accession numbers JX294920-JX294923.

Results

Analysis and comparison of nucleotide and deduced amino acid sequences of the HA genes. The nucleotide sequence of the HA gene comprised 1683 nucleotides encoding a protein of 560 amino acids, which includes complete receptor-binding, cleavage and glycosylation sites. None of the isolates exhibited insertions or deletions within this region upon comparison with the HA gene of the H9N2 subtype prototype (A/turkey/Wisconsin/66). However, rather numerous instances of nucleotide substitution point mutations within the gene were observed. Amino acids were numbered according to the residue positions in the HA of the human H3 subtype of A/Aichi/2/68 (H3 numbering) and turkey H9 subtype of A/turkey/Wisconsin/66 (H9 numbering) prototype strains.

The amino acids in the HA cleavage site of all 4 recent isolates possess a PAKSSR/GL motif, which was the same as those of ck/IR/EBGV-88/10 (unpublished data, GenBank accession number: JN646748), ck/IR/N101/11 and ck/IR/N102/11 (unpublished data, GenBank accession numbers: JQ970436 and JQ970437), that were previous isolates from Iran. The isolates from Egypt in 2011 (ck/EG/S4456B/11) and Pakistan in 2008 (ck/PK/UDL01/08) also had a similar motif in their HA cleavage sites.

All the 4 recent isolates from this study possessed histidine (H), alanine (A), leucine (L) and isoleucine (I) at positions 183, 190, 226 and 227 respectively, which are the key residues in receptor-binding sites (H3 numbering). Interestingly, all recent isolates, which were obtained from different parts of the Tehran province during an epidemic, possessed the same amino acids at these positions. Table 1 presents the amino acids at the cleavage and receptor-binding sites of the HA genes of some H9N2 viruses.

The analysis of the potential glycosylation sites in the HA gene of these 4 recent viruses revealed eight common sites with the N-X-T/S motif (in which X may be any amino acids except proline) in the sequenced part of the HA gene; six of them were located in the HA1 protein, and the other two in the HA2 protein of the molecule. The

positions were at 29-31 (NST), 105-107 (NGT), 141-143 (NVT), 298-300 (NST), 305-307 (NIS), 492-494 (NGT), and 551-553 (NGS) (H9 numbering).

Table 1. Amino acids at key residues of cleavage and receptor-binding sites of HA genes of some H9N2 viruses

	Cleavage site				Receptor binding site			
	326	327	328	329	183	190	226	227
A/chicken/AGH-B1/2012 (H9N2)	K	S	S	R	H	A	L	I
A/chicken/AGH-B2/2012 (H9N2)	-	-	-	-	-	-	-	-
A/chicken/AGH-B3/2012 (H9N2)	-	-	-	-	-	-	-	-
A/chicken/AGH-B4/2012 (H9N2)	-	-	-	-	-	-	-	-
A/chicken/Iran/N102/2011 (H9N2)	-	-	-	-	-	-	-	-
A/chicken/Iran/EBGV-88/2010 (H9N2)	-	-	-	-	-	T	-	-
A/chicken/Iran/SS2/2008 (H9N2)	R	-	-	-	-	-	-	-
A/chicken/Iran/THLBM862/2007 (H9N2)	R	-	-	-	-	-	-	-
A/mallard/Iran/T366/2007 (H9N2)	A	-	D	-	-	E	Q	Q
A/chicken/Iran/B102/2005 (H9N2)	R	-	-	-	-	-	-	-
A/chicken/Iran/B308A/2004 (H9N2)	R	-	-	-	-	-	-	Q
A/chicken/Iran/B314/2003 (H9N2)	R	-	-	-	-	-	Q	Q
A/chicken/Iran/233/2001 (H9N2)	R	-	-	-	-	-	-	Q
A/chicken/Iran/565/2000 (H9N2)	R	-	-	-	-	-	Q	Q
A/chicken/Iran/705/1999 (H9N2)	R	-	-	-	-	-	Q	Q
A/Chicken/Iran/TH77/1998 (H9N2)	R	-	-	-	-	E	-	Q
A/chicken/Pakistan/CP/2010 (H9N2)	-	-	-	-	-	-	-	-
A/avian/Saudi_Arabia/910134/2006 (H9N2)	R	-	-	-	-	T	-	-
A/Hong Kong/1074/1997 (H9N2)	R	-	-	-	-	E	-	Q
A/Quail/Hong Kong/G1/97 (H9N2)	R	-	-	-	-	E	-	Q
A/turkey/Wisconsin/66 (H9N2)	V	-	-	-	-	E	Q	Q

Amino acids' abbreviations: K = Lysine, S = Serine, R = Arginine, H = Histidine, A = Alanine, L = Leucine, I = Isoleucine, T = Threonine, D = Aspartic acid, E = Glutamic acid, Q = Glutamine, V = Valine

Phylogenetic analysis. The phylogenetic tree (Fig. 1) illustrates three distinct groups. Group 1 consists of the Middle East strains and some strains from Nepal, India and Hong Kong. The isolates from this study, along with the other two Iranian strains from 2010 and 2011 (ck/IR/EBGV-88/10 and ck/IR/N102/11) (unpublished data), and also four Pakistani strains isolated during 2005 to 2008 (ck/PK/UDL03/05, ck/PK/UDL04/06, ck/PK/UDL04/07 and ck/PK/UDL01/08), comprise one of the branches of group 1. Furthermore, the tree group 1 (Fig. 2) consists of three distinct clades. Some of the Iranian isolates and some other isolates from Israel, Egypt, Lebanon, Saudi Arabia, Pakistan, Iraq, India and also Nepal, comprise the first clade of group 1. Previous Iranian strains

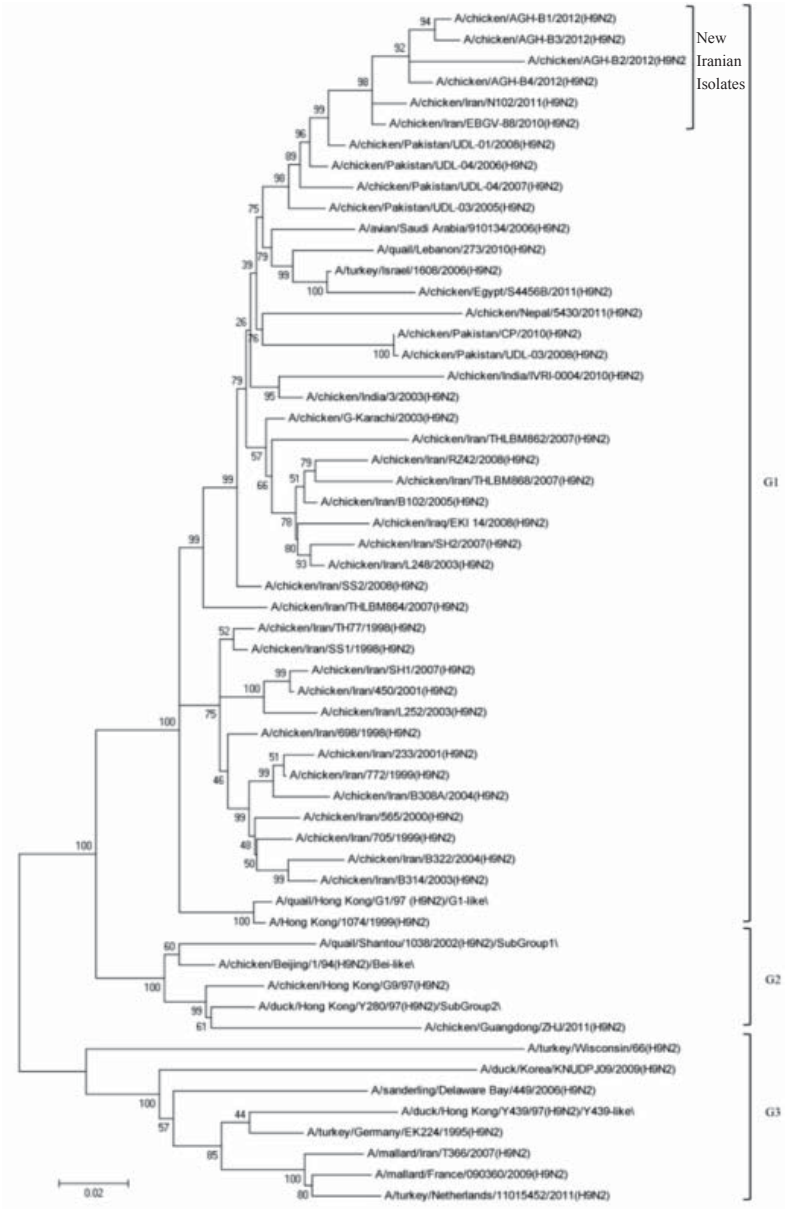

Fig. 1. Phylogenetic tree for the hemagglutinin genes of 4 isolates in this study and 53 selected isolates

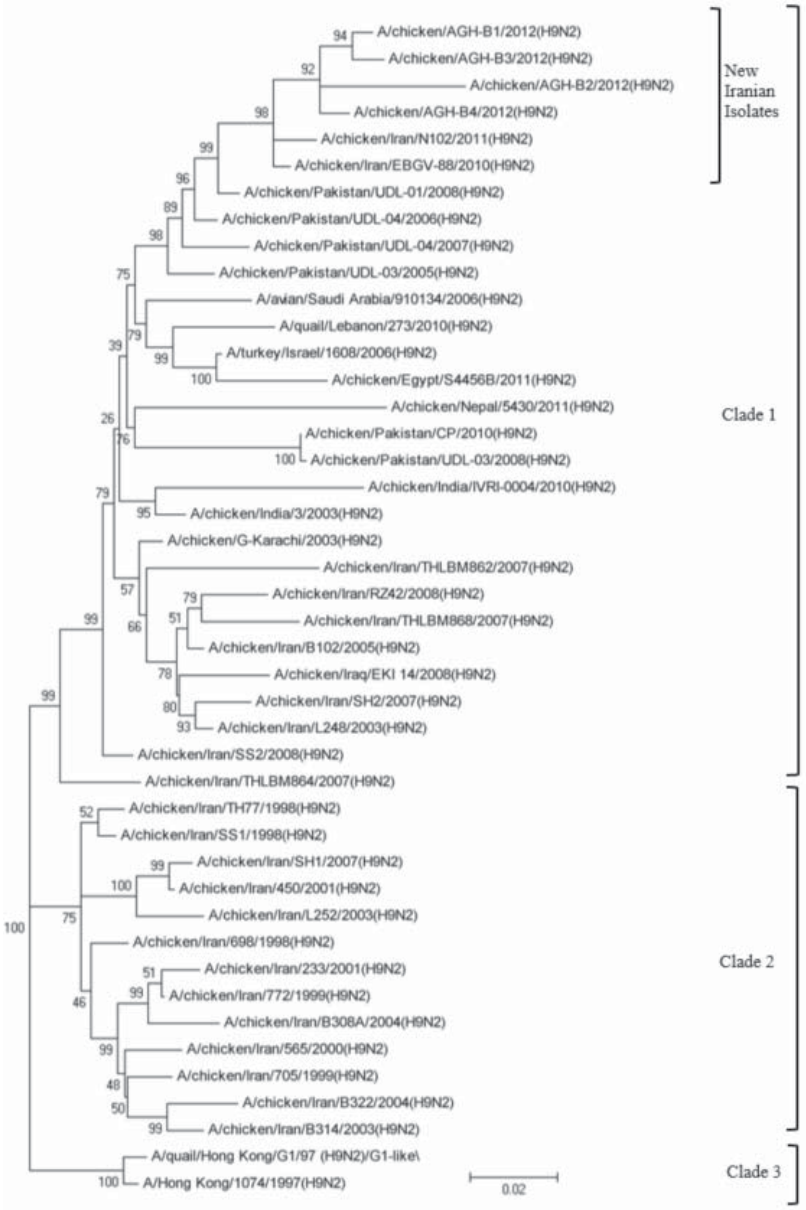

Fig. 2. Phylogenetic tree for the hemagglutinin genes of group 1 H9N2 influenza viruses used in this study

from the earliest outbreaks in 1998 and some of the strains isolated in Iran before 2007, also comprise the second clade of group 1; besides this clade, human H9N2 from Hong Kong, and qu/HK/G1/97 form the third clade of group 1.

Group 2 consists of the mainland China and Hong Kong strains. Group 3 is constructed from the USA and Europe isolates, one isolate from Hong Kong, as well as the isolates from wild migratory birds from the USA, Europe and even Iran.

Analysis of percent identity and divergence among nucleotide sequences of the HA genes. Table 2 presents the comparison of nucleotide sequence homology among the HA genes of the isolates from this study, and also some other H9N2 isolates.

Table 2. Comparison of nucleotide sequence homology of 4 new isolates with some others (%)

	4 new isolates	Iranian isolates	HK/1074/97*	HK/35820/09*	Qu/HK/G1/97
4 new isolates	96.0-99.1	83.8-99.1	89.1-89.4	85.9-86.1	87.7-88.0
Iranian isolates		81.6-99.1	85.2-95.1	83.7-92.7	87.5-94.8
HK/1074/97*				90.8	94.5
HK/35820/09*					92.0
Qu/HK/G1/97					

* Isolated from human influenza cases during outbreaks of the disease in Hong Kong in 1997 and 2009

Discussion

The molecular determinants of pathogenicity and virulence of the HA protein are the cleavage site peptide sequence, specific amino acid residues at the receptor-binding sites, and the presence or absence of glycosylation sites around the receptor-binding sites (TOMBARI et al., 2011).

The pathogenicity of H5 and H7 AI viruses is associated with the presence of multiple basic amino acids at the cleavage site of HA. Kosuke Soda et al. demonstrated that an H9N2 virus has the potential to acquire intravenous pathogenicity in chickens when a pair of di-basic amino acid residues is introduced at the cleavage site of its HA protein, although the morbidity via the nasal route of infection was still lower than that of H5N1 HPAIV (SODA et al., 2011). All 4 recent isolates from this study possessed a PAKSSR/GL motif at the cleavage site of the HA, just like the Iranian isolates from 2010 and 2011 (ck/IR/EBGV-88/10 and ck/IR/N102/11) (unpublished data), while this motif was not the same as those in previous isolates from Iran and the Middle East before 2008, qu/HK/G1/97, ck/HK/G9/97 and human H9N2 of Hong Kong (HK/1074/97 and HK/35820/09). However, some recent isolates from the Middle East, such as three viruses isolated from Pakistan in 2008 and 2010 (ck/PK/UDL01/08, ck/PK/UDL03/08 and ck/PK/CP/10), and also one isolate from Egypt in 2011 (ck/EG/S4456B/11), possessed the PAKSSR/GL

motif in the cleavage site, while the previous UDL isolates from Pakistan in 2005-2007 possessed the PARSSR/GL motif in this site. Moreover, in South Asia, two isolates from India in 2010 (ck/IN/IVRI-0004/10) and Nepal in 2011 (ck/NP/5430/11) possessed the PAKSSR/GL motif in the cleavage site of the HA.

During 1998 to 2009, the PARSSR/GL motif at the cleavage site of the HA was predominant among the Iranian isolates (KARIMI et al., 2004; KARIMI et al., 2008; KIANIZADEH et al., 2006; TOROGHI and MOMAYEZ, 2006) except for a few cases; FEREIDOUNI et al. (2010) reported the PAASDR/GL motif at the cleavage site of the H9N2 isolate from a mallard duck in Iran in 2007 (mld/IR/T366/07) and GHADI et al. (2009) detected PARSNR/RL and PARSNK/RL motifs at this position in two isolates from live bird markets in the north of Iran in 2007 (ck/IR/THLBM867/07 and ck/IR/THLBM868/07). Furthermore, GHALYANCHI LANGEROUDI et al. (2012) reported PARSNR/GL and PTRSSR/GL motifs at the cleavage site of two isolates from Iran (ck/IR/TH386/07 and ck/IR/TH85/07), which were similar to the ones of the isolates from Israel (FEREIDOUNI et al., 2010; GHADI et al., 2009; GHALYANCHI LANGEROUDI et al., 2012).

All 4 recent isolates from this study showed the PAKSSR/GL motif at the cleavage site of the HA, where in fact a basic amino acid, arginine (R), was replaced by another basic amino acid, lysine (K), in this site in comparison to the previous isolates; but the importance of this substitution is still not clear. However, these results may reflect the wide circulation of the virus in the country and the region. This motif indicates the low pathogenic nature of the recent H9N2 strains and also their potential to acquire the basic amino acids required for highly pathogenic strains through the addition of a single basic amino acid in this site (GUO et al., 2001).

It has been well-documented that amino acids in the receptor-binding sites of HA are critical for receptor specificity of HA, which plays an important role in determining the host range of influenza viruses (GAMBARYAN et al., 2002; HA et al., 2001). In this study, the key residues in the receptor-binding sites of HA were studied, with a comparative approach to the previous AI isolates. In general, AI viruses preferentially recognize sialyl-α2,3-galactose receptors, while human viruses recognize sialyl-α2,6-galactose receptors. As has been shown previously, all HA proteins of influenza A viruses isolated from avian species possess H, E and Q at positions 183, 190 and 226 in the receptor-binding sites respectively (MATROSOVICH et al., 2001; MOOSAKHANI et al., 2010). In contrast, isolates from the present study possessed A at position 190 and L at position 226.

Position 190 is reported to influence the affinity of binding to the sialyl-α2,6-galactose linkage. The binding affinity is at the weakest level with A at position 190. Also, it is at the intermediate level with T, and at the highest level with V at the same position. In 1918, the substitution at position 190 generated the H1 human pandemic strain (MATROSOVICH

et al., 2001; MOOSAKHANI et al., 2010). At this position, all 4 recent isolates from this study, just like all UDL isolates from Pakistan in 2005-2008 (ck/PK/UDL03/05, ck/PK/UDL04/06, ck/PK/UDL04/07, ck/PK/UDL01/08, ck/PK/UDL03/08), possessed A (IQBAL et al., 2009). Furthermore, most of the Iranian isolates possessed A at this position, except for ck/IR/EBGV-88/10 with T and also mld/IR/T366/07, ck/IR/TH77/98 and ck/IR/SS1/98 with E at this position.

L at position 226 of these viruses is common in the sequence found in the HA proteins of influenza virus subtypes H2 and H3 isolated from humans (MATROSOVICH et al., 2000). The HA possessing L-226 binds to the sialyl-α2,6-galactose linkage, and Q-226 binds to the sialyl-α2,3-galactose linkage (MATROSOVICH et al., 2000; MOOSAKHANI et al., 2010). The Q to L substitution at position 226 allows H9N2 viruses to replicate more efficiently in human cells in culture (WAN and PEREZ, 2007). All 4 recent isolates from this study, those from the United Arab Emirates (UAE), isolated by Aamir in 2000 (qu/Dubai/301/00 and qu/Dubai/303/00) and also UDL isolates from Pakistan in 2005-2008, possessed L at position 226 (AAMIR et al., 2007; IQBAL et al., 2009). Most of the Iranian isolates also possessed L at this position, except for mld/IR/T366/07, ck/IR/B314/03, ck/IR/565/00, ck/IR/705/99 and ck/IR/772/99, which possessed Q at this position.

The 4 recent isolates from this study, just like the isolates from the UAE (qu/Dubai/301/00 and qu/Dubai/303/00) and Pakistan (UDL isolates from 2005 to 2008), contain the amino acid residue I at position 227 within the receptor-binding sites. Although I at position 227 was predominant among the Iranian H9N2 isolates during 2005-2012, except for two isolates (ck/IR/SH1/07 and mld/T366/07), most of the Iranian strains isolated during 1998-2004 possessed Q at this position, except for ck/IR/L248/03 with I at this position. However, detailed structural analysis of the HA gene receptor-binding sites is still required to resolve the importance of the amino acids at these positions (AAMIR et al., 2007; IQBAL et al., 2009).

The number of potential glycosylation sites of HA of H9N2 viruses varies from six in duck isolates to eight in chicken and quail isolates. The consensus amino acid sequences of all 4 recent isolates from this study revealed eight potential glycosylation sites in the HA protein. One potential glycosylation site at position 218-220 (NRT) (H9 numbering) of the isolates was lost in comparison to the H9N2 viruses isolated from birds in the Middle East in 2000-2003, ck/IR/SS1/98, ck/IR/SH1/07 and mld/IR/T366/07, and also the human H9N2 isolates from Hong Kong in 1997 (HK/1074/97) (FEREIDOUNI et al., 2010; HOMAYOUNIMEHR et al., 2010; SHAHSAVANDI et al., 2012). The loss of potential glycosylation sites may represent an adaptation of H9N2 within poultry (IQBAL et al., 2009). GHALYANCHI LANGEROUDI et al. (2012) showed that deletion of one amino acid residue at positions 298-300 in ck/IR/TH286/07 and 492-494 in ck/IR/TH79/00 isolates from Iran led to the loss of one potential glycosylation site on the HA protein

(H3 numbering) (GHALYANCHI LANGEROUDI et al., 2012). GHADI et al. (2009) also suggested that a similar deletion in the live bird markets isolates from the north of Iran, may represent mutations that could have occurred in the markets (GHADI et al., 2009).

The results of the phylogenetic study in this paper are basically in accordance with previous publications, with minor variations. Genetic and phylogenetic analysis of the HA genes showed that new H9N2 strains isolated from chickens in Iran during 2010-2012 formed a distinct subgroup of the previous strains. Previous studies showed that the isolates from Pakistan, Iran, and Saudi Arabia revealed a very close relationship and may have a common source. Although all viruses forming group 1 in previous studies were only from the Middle East and Hong Kong (HK/1073/99, HK/1074/97 and qu/HK/G1/97), the presence of strains from Nepal and India in this group in this study is interesting and may show a common origin of these viruses. It may also show the wide circulation of these viruses in the Middle East and South Asia.

In the present study, like previous ones, the isolates from Hong Kong and China formed one lineage (group 2), and the isolates from the USA, Europe, Korea, one isolate from Hong Kong and even Iranian isolates from migratory wild birds, formed a separate lineage (group 3). The unexpected presence of Korean, Hong Kong and Iranian isolates from migratory wild birds and ducks in this group may show the importance of these birds in the movement of the viruses across the continents.

The highest and lowest percent identity among the 4 recent isolates from this study were 89.4 and 89.1 respectively (Table 2). Also, there was no high identity among these isolates and the previous Iranian isolates. The highest percentage identity in the case was between ck/IR/AGH-B4/12 and ck/IR/EBGV-88/10 (95.2 %). CAMERON et al. (2000) reported that the HA genes of the viruses isolated in the earliest outbreaks in Iran during 1998-1999 were closely related to the HA genes of the human H9N2 and qu/HK/G1/97 viruses isolated in Hong Kong (CAMERON et al., 2000). In the present study, there was no high identity between the recent Iranian isolates and Hong Kong human H9N2 viruses, HK/1074/97 and HK/35820/09 (89.1-89.4 % and 85.9-86.1, respectively). It is important to note that there was lower percent identity between the isolates from this study and the Honk Kong human H9N2 of 2009 (85.9-86.1) in comparison to the human isolate from Hong Kong in 1997 (89.1-89.4). The highest percent identity between all Iranian isolates and the human H9N2 viruses from Hong Kong was between ck/IR/TH77/98 and HK/1074/97 (95.1 %) (Table 2).

The results of the present study show the changing nature of H9N2 AI viruses circulating in poultry farms of the Tehran province, and may be used as a reference for further studies. Recent changes in the H9N2 viruses may be the result of the widespread circulation of these viruses in recent years. This may be due to the lack of adequate surveillance and control programs, such as vaccination and quarantine. Although

vaccination is an important tool to control epidemics, the use of vaccination might cause an outbreak of a vaccine-resistant strain due to increased immune pressure on AI field viruses. Since the virus is constantly changing, development of new vaccines based on protection studies is recommended on a regular basis, to overcome the vaccine-induced immune pressure. Furthermore, more research may be needed on the use of antigens derived from the very first outbreak's strains, which are widely used for routine LPAI screening in Iran, and recent isolates for assessing HI titer, especially in order to check the immunity against the new circulating strains. Hence, it is highly recommended that continuous surveillance programs and genetic analysis of ongoing changes to the H9N2 should be considered.

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GHORBANI, A., F. MOOSAKHANI, M. V. MARANDI: Filogenetska analiza gena za hemaglutinin podtipa H9N2 virusa influence ptica izdvojenih iz tovnih pilića u Iranu. Vet. arhiv 86, 95-109, 2016.

SAŽETAK

Istražene su inačice gena za hemaglutinin četiriju nedavno izdvojenih izolata podtipa H9N2 virusa influence ptica na području Teherana u Iranu. Geni su umnoženi i sekvencirani s ciljem da se obilježja virusa usporede s prijašnjim izolatima iz Irana i nekih drugih zemalja u svijetu. Analizirani su geni za hemaglutinin u punoj dužini od 112 izolata H9N2 iz pilića i ptica selica iz cijelog svijeta, uključujući i 68 izolata iz Irana od 1998. do 2012. Aminokiselinski sastav na mjestu cijepanja hemaglutinina svih četiriju nedavno izdvojenih izolata sadržavao je PAKSSR/GL motiv, koji je bio različit u usporedbi s prevladavajućim PARSSR/GL u hemaglutininu iranskih sojeva H9N2 izdvojenih prije 2010. Sva četiri izolata posjedovala su histidin, alanin, leucin i izoleucin na pozicijama 183, 190, 226 i 227, koje su ključne za vezanje na receptore. Genetska i filogenetska analiza gena za hemaglutinin pokazala je da izolati H9N2 iz pilića u Iranu izdvojeni od 2010. do 2012. čine posebnu i različitu podskupinu od prijašnjih izolata. Dokazane izmjene tih izolata mogle bi biti uzrok njihova širenja posljednjih godina. To se moglo dogoditi zbog nedostatka odgovarajućeg programa nadzora i kontrole koji bi obuhvaćali cijepljenje i karantenu. Stoga se posebice preporučuje provođenje programa genetske analize te trajnog praćenja trenutnih promjena podtipa H9N2.

Ključne riječi: virus influence ptica, H9N2, hemaglutinin, filogenetska analiza, Iran