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Original Article

Amino Acid Sequence Analysis of Hemagglutinin Protein of H9N2 Isolated from Broilers in Tehran in 2007

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Abstract

Background and Aims: Since 1998, Iranian poultry industry has been affected by avian influenza (AI) virus, subtype H9N2. The association of high mortality and case report of H5N1 and H9N2 influenza virus in wild birds in recent years raised the suspicion of a possible new genetic modified AI virus.

Methods: Partial nucleotide sequences and deduced amino acid of hemagglutinin (HA) genes of 4 H9N2 influenza viruses isolated from broilers in Iran in 2007 were genetically analyzed.

Results: The isolates possessed two types of amino acid motif -R-S-S-RlG-L- and -R-S-N-RlG-L- at the cleavage site of HA. "-R-S-N-RlG-L in Iranian isolates is the same as the motif previously reported in Israel. Glycosylation site of the virus has been missed in some isolates. Receptor binding site of the isolates were found similar to the human H9N2 isolates.

Conclusion: Based on published reports for high similarity of H9N2 with human H5N1 isolates, it is important to consider the potential of Iranian avian influenza viruses to infect human.

Keywords: Avian influenza virus; H9N2; Chicken; Hemagglutinin; Cleavage Site

Introduction

Avian influenza (AI) is a highly contagious disease with significant potential to harm poultry industry often resulting in extensive losses (1). Influenza viruses belong to the Orthomyxoviridae family of segmented negative-strand RNA viruses divided into five genera: Influenza A, B, and C virus, Thogotovirus and Isavirus (2,3). The hemagglutinin (H) and neuraminidase (N) are two structurally distinct envelope glycoproteins inserted into the lipid bilayer to form the surface of the virion. HA is the maj or antigen for neutralizing antibodies and is involved in binding of virus particles to receptors on host cells (2, 3). The antigenic differences of hemagglutinin and neuraminidase of the viruses provide the basis of their classification into subtypes. Serologically 16 Hand 9N subtype of AI viruses has been isolated in chicken (2,4).AI outbreaks in industrial chickens and turkeys are characterized by the sign of respiratory distress, decreased egg production and mortality rates that ranges up to almost 100%. Field evidence shows that a variety of pathogenicity characteristics, poorly defined host and environmental factors also play an important role in determining the outcome of the infection (3). Since 1998, H9N2 AI outbreaks have been one of the major problems in Iranian poultry industry (5-7). Mortality rate of H9N2 influenza virus outbreak in broilers chicken farms during 19982001 in Iran was 20-60% (8).

Euro-Asia H9N2 isolates are represented by three groups: A1DucklHongKong/Y280/97 *(A1DklHK/Y* 280/97) and A1Quail/Hong Kong/G 1/97 (A/Qa/HK /G 1/97) (9), A1Qa/HK/G 1/97 is

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thought to be the donor of 6 "internal" genes to the poultry and human H5N1 viruses isolated in 1997 (10). Documented cases of human infection with H9N2 avian influenza viruses, first detected in 1999 in Hong Kong and China, indicated that these viruses can be directly transmitted from birds to humans (11). Previous studies have documented the ability of H9N2 viruses to use receptor and binding characteristics that are similar to that of the human pandemic influenza viruses (12). Thus H9N2 influenza viruses are considered to be one of candidates for the next pandemic (11, 12). Pathogenicity of the influenza viruses is caused by many genes, but the role of hemagglutinin, particularly its cleavage site, is the most important. Hemagglutinin is synthesized intracellularly as an inactive protein-precursor (HAO). In the course of the infection, HAO is cleaved into two disulfide-linked polypeptide chains, HAl and HA2 (13). The cleavage site of 13 from 15 HA subtypes (all HA subtypes except H5 and H7) consist of four amino acids including a single arginine residue which is removed from the COOH terminus of the HAl after cleavage by means of virus-associated carboxypeptidase (14). A correlation between cleavability of the HAO and pathogenicity has been established. The HAO protein of non-pathogenic influenza viruses can be cleaved extracellularly by trypsin-like enzymes originating from co-infecting bacteria or host inflammatory response induced by respiratory tract infections (15, 16). Influenza viruses can also predispose the host to secondary bacterial infections by causing tissue damage, inhibition of bacterial clearance, promotion of bacterial adherence, and interference with nonspecific immunity. The cleavage is fulfilled mainly by extracellular serine proteases recognizing Q/E-X-R motif In a few cases of the H5 and H7 subtypes, in which polybasic sequences are inserted between HAl and HA2 chains, the cleavage is carried out by the intracellular subtilisin-like enzymes, which recognize R-XR/K-R motif However, R-X-R/K-R motif presence in the cleavage site is not sufficient for successful cleavage; it has been shown that the cleavage site of the majority of influenza viruses (consisting of four amino acids) is inaccessible for the action of intracellular furin-like proteases. A cleavage site becomes accessible for intracellular proteases after either insertion of at least two additional basic amino acids, or after removing lateral carbohydrate chain (15-18). The cleavage site amino acid sequence is specific in each region.

All types of this motif have been shown in table 1. (19) Another site in this region that should be studied is the left edge of receptor amino acid that covers amino acids 224-229. In this region we have two positions 206 and 208 that is very important in attachment to human receptors (11, 12). The association of high mortality in recent years and report of H5N1 and H9N2 in wild birds in Iran (20) raised the probability of a possible new genetic modified AI virus. In this study, we analyzed nucleic acid as well as amino acid sequence of the cleavage site of hemagglutinin protein of H9N2 Iranian isolates obtained from poultry in Tehran, Iran during 2007.

Methods

Sample Collection

Sample collection from lung & intestine of suspected infected broiler in Tehran province was performed according to the standard method in a 2X phosphate buffer solution (PBS, pH 7.4) containing 10.000 IV/ml Penicillin, 1 mg/ml Streptomycin Sulphate, and 20IV/ml Nystatin . Specimens were stored at -70°C for further testing.

Virus Isolation

Ten-day-old embryonated chicken eggs were inoculated and incubated at 37 °C for 48 h. The eggs were candled daily and embryos dying within 24 h post inoculation were discarded. Allantoic fluid was harvested and presence of the virus was determined by hemagglutination assay. The identification of the virus subtype was determined by standard hemagglutination inhibition (HI) tests using polyclonal chicken antisera. The allantoic fluids containing virus were harvested and stored at -70°C for further use. All of HA negative allantoic fluids were inoculated for the second passage (21).

RNA Extraction

Viral RNA was extracted from infected allantoic fluid using RNX reagent according to the manufacturer's instruction. Briefly, in an RNAase free 1.5ml tube, 800/-11 of RNX TM-Plus solution (Cinnagen, Iran) was added to 200/-11 allantoic fluids. After shaking, 200/-11 of chloroform was added and the mixture was

centrifuged at 14,000 rpm at 4°C for 15min. Equal volume of Isopropanol was added to the upper phase in a new tube. The mixture was centrifuged at 12,000 rpm at 4°C for 15min. The supernatant was discarded and 500/-11 of 75% ethanol was added to the pellet. After centrifugation at *7,500g* for 10min at 4°C, the supernatant was discarded and the pellet was dried at room temperature for few minutes. Finally, the pellet was diluted in 20/-11 DEPC water. To help dissolving, the tube was incubated at 55-60°C in water bath for 10 minutes and stored at -70°C for RT-PCR reaction (22).

RT and PCR reaction

Reverse transcription was done by using oligonucleotide influenza universal primer unil2: 5-AGC AAA AGC AGG-3 with "Revert Aid" first strand cDNA synthesis Kit (Fermentas, Canada). Amplification of the HA gene was carried out by PCR as described previously using one pair of specific primers (23). Primer sequences are available upon request. The reaction mixture (50/-11) contained 5/-11 of cDNA, 15 pmoles of forward and reverse primers (4/-11), and Cinnagen master mix (Cinnagen, Iran). The amplification protocol was: one step of denaturation at 94 °C for 3 min, 35 cycles of 94 a C *1* 45 Sec, 58 °C *1* 45 Sees, and *noc 160* Sees, and final extension at *n* "C for 10 mins. The PCR products were separated by electrophoresis using 1 % agarose gel. PCR products were purified with the QIA quick Gel Extraction Kit (Qiagen, Valencia, CA, USA) (23).

TA cloning and sequencing

Purified PCR products for sequencing were cloned into plasmid for TA cloning with *Ins T/A* cloning kit (Fermentas, Canada) according to the manufacturer's instruction .Plasmid extraction from positive clone was carried out by QIA miniprep plasmid extraction kit (Qiagen, Valencia, CA, USA). Following digestion with ECORI (F ermentas, Canada) to confirm the insertion, the nucleotide sequences were analyzed by plasmid sequencing on an automated 3700 DNA sequencer (Applied Biosystems, Foster city, CA, USA).

TH286 (99%). The isolates had high similarity with Iranian isolates (AB0099I4 & AB00992I) from 2003. But homology with other Iranian isolates obtained earlier was less (data have not been shown).

Sequence analysis of the haemagglutinin cleavage site

Nucleic acid was extracted from the isolated viruses and subjected to nucleotide sequencing of the genome coding for the cleavage site of the haemagglutinin molecule. Initially, nucleotide and deduced amino acid sequences were aligned using Free CLC Bio workbench program. Sequence data were compiled and analyzed using sequence analysis software package (DNASTAR). Multiple nucleotide and amino acid sequence alignments were generated using Web-based software in Influenza source information (NCBI).

Nucleotide Sequence Accession Numbers

The nucleotide sequences for H9N2 influenza viruses used in this study are available at GenBank under accession numbers EU477242 through EU477245. The accession numbers are provided in figure 1.

Results

Homology of amino acid sequence

The maximum amino acid sequence homology in this study has been observed between THI86 and Amino Acid Sequence analysis of A/H9N2 HA cleavage site. The amino acid sequence (180-360) of HA were deduced from the nucleotide sequence. Four new Iranian isolates were compared with other published Iranian isolates and others H9N2 subtypes from different subgroups (figure. 1). Amino acid sequences at the cleavage site of the HA of the isolates possessed -P-A-R-S-S-R/G-Lmotif, except two isolated: TH85 (A to T) and TH386 (R to N). Amino acid changes in these two isolates were not significant. All changes in region of Nucleotide sequence that codes cleavage site are available in figure 3. Some differences were found at positions: 183(S to D), 204 (I to T), 216(D to N), 218(N to D), 224(V to L), 271(G to E), 283(S to N), 313(N to T), 318(V to I), 319(R to G), 327(V to I).

Table 1. Cleavage site motif variations among H9 influenza viruses in the world (19).

z	Cleavage site motif	Motif	Virus strain	Genbank accession number
	ALDR		Alduck/Hong Kong/366/78	AY206674
2	ASAR	L	Alturkey/Gennany/R33/96	AJ781821
3	ASDR	C	Alduck/Hong Kong/784/79	AFI56383
4	ASGR	B	Alduck/Hong Kong/l68/77	AFI56382
5	ASNR	J	Alduck/Hong Kong/l47/77	AY20667I
6	ASYR		Alostrich/South Africa/9508103/95	AF508554
7	GSSR	M	*AI* chicken!GuangdongiSS/94	AF384557
8	ISGR	H	Alturkey/Italy/l25/89	AF218095
9	ISNR	N	Alpekin duck/Singapore/F91-5/9/97	AF218105
10	KSSR		*AI* chicken! Jiangsu/2/9 8	AF461510
II	RLSR		Alsw/ShanDong/I/2003	AY294658
12	RSKR		Alturkey/Givat Haim/868/02b	DQ10447 I
13	RSNR		Alchicken!Sede Uzziyyahu/1651104b	DQ108923
14	RSSR	K	AlHong Kong/l 073/99b	AB080226
		O	Alavian!China/417-428/98	AF218106
15	TSGR	E	Alturkey/ItalyIVR86/83	AF218088
16	TSNR	F	Alturkey/Itali/24 5 /84	AF218106
17	VSDR	D	Alduck/Hong Kong/448/78	AB080224
18	VSNR	G	Alduck/Hong Kong/702/79	AY206672
19	VSSR	A	Alduck/Iudia/31 g/86	AF218091

Left-edge of binding pocket

This region is amino acid residues at position 224229 that been marked in figure 1&2. Left-edge of binding pocket as one marker should be checked in H9N2 virus. Except TH85, other recent Iranian isolates have (NGLIGR) motif.

Discussion

In recent years, AI has caused maj or economic harms in Iranian poultry industry. The latest Iranian H9N2 isolate has been reported to have low pathogenecity for SPF chickens. However, H9N2 outbreaks have caused up to 65% mortality rate in commercial chicken and are the only influenza subtype in the chicken population in Iran (5-8). Although pathogenecity of avian influenza virus depends on many factors, the amino acid sequence of cleavage site ofHA is considered to be its major determinant. The pathogenecity of H5 and H7 influenza viruses is related to basic amino acids at cleavage site of HA.

It is not clear whether amino acid sequences at the cleavage site of the HA of H9N2 subtype virus is related to its pathogenecity, although it is similar to the motif (R-X-R1K-R) required for highly pathogenic viruses of H5 and H7. Only one nucleotide substitution could change the -R-S-S-Rmotif to -R-S-R-R-. it was (13-18) suggested that H9N2 viruses isolates in Iran are potentially capable of becoming highly pathogenic. The presence of Serine amino acid has made it a crucial motif because only 1 nucleotide substitution (C to A or G) at two positions is enough to convert this motif to a virulent sequence. To determine the epidemiological characteristics and the HA cleavage site motifs of the Four Iranian H9N2 isolates, the corresponding segments sequences comprising the HA cleavage site were studied and compared to those from other H9N2

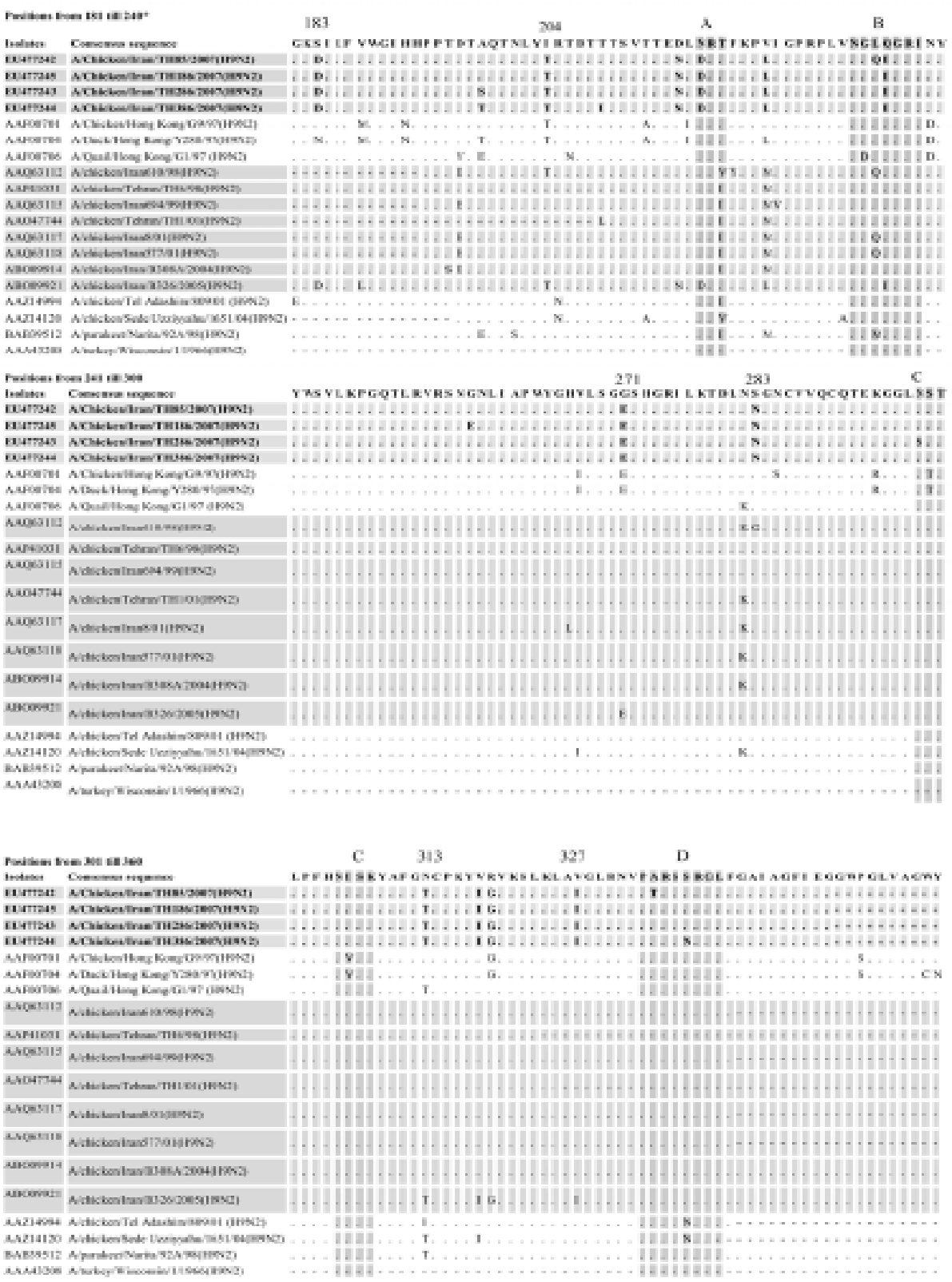

Fig. 1. Comparison of amino acids (residues 181-360) among this study isolates with other H9N2 isolates. A: Missed Glycosylation site
B: Left-edge of binding pocket
C: Glycosylation site
D: Cleavage site
* Amino acid positions are numbered according to the Nturkey / Wisconsin /66

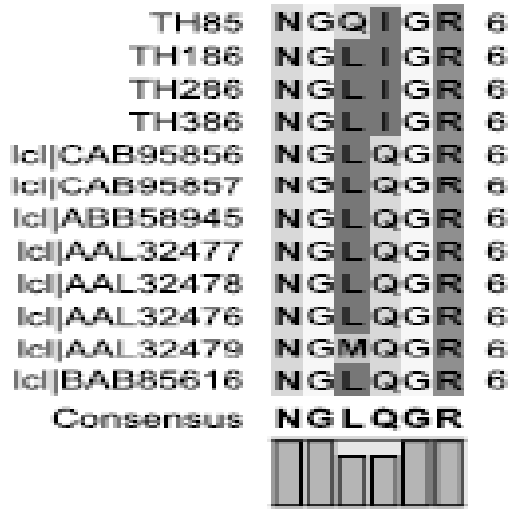

Fig. 2. Comparison of Left-edge of binding pocket's amino acid sequence of Iranian H9N2 isolates in this study with H9N2 viruses isolated from human host.

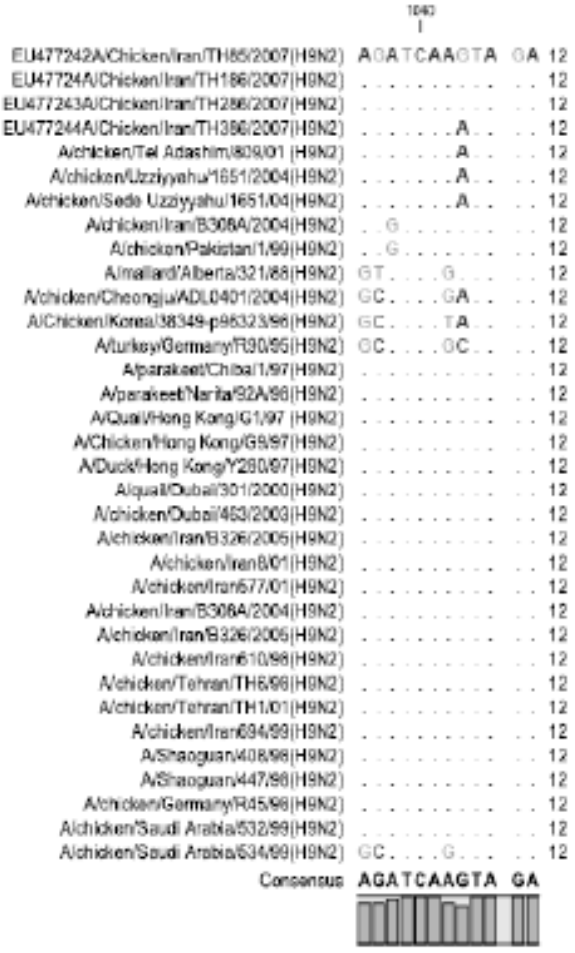

Fig. 3. Nucleotide sequences of the H9N2 viruses at cleavage site region to compare the isolates of this study with some other isolates.

isolates. Comparison of the HA cleavage site motifs of all the isolates indicated a common motif (PARSSRG) with two exception: TH85 (A to T) and TH386 (S to N). None of the 4 different motifs contained multiple basic amino acids. Cleavage site motif in TH386 is similar to isolates of Israel such as AAZ14994 and AAZ14120. PARSNRG motif is specific to isolates of Israel (19). This motif did not exist in Iranian isolates prior to 2007. Amino acid in cleavage site of TH85 (PTRSSRG) is found in isolates with accession number AAL65235 and ABV46459 .These isolates were similar to Chinese isolates. During 10 years of observation, -R-S-SR-, motif predominated amongst the Iranian isolates (24-27), new motifs also appeared among the Iranian isolates. It is the first report of emergence of new substitution of amino acid in cleavage site of HA from Iran. However, the presence of this particular motif also emphasizes that these viruses have the potential to become pathogenic, should they acquire any further amino acid substitutions in the HA-connecting peptide region. Some of the positions differences founded in the isolates of this study, had not been detected in Iranian isolates prior to 2004.

Study about Left-edge of binding pocket, reveal that three isolates in this study (TH186, TH286 and TH386) have motif similar to H9N2 viruses isolated from human samples (Figure 3). This finding alert this subject that Iranian isolates have tendency to infect humans. This kind of motif had been shown in other Iranian isolates that were obtained from Genbank. One chicken isolate (TH85) from Tehran in 2007 contained a Q-234 instead of L-234 in HAl receptor binding site therefore it did not have the potential to infect humans. L-234 substitution was seen in some sequence of HA gene of Iranian H9N2 isolates and recently isolated (TH186, TH286, TH386), thus these isolates can bind to human receptors (a-2, 6) and may infect humans populations (2, 12). This substitution was seen in some sequence of HA gene of Iranian H9N2 isolates. Amino acid 198 (Receptor binding site of HA) in one isolate (TH286) conserved to the new motif in Iranian isolates (28, 29).

Analysis of the HA gene of H9N2 viruses revealed seven glycosylation site with the *NXT/S* motif (X can be any amino acid except proline), five in HAl and two in HA2. Based on these results, in TH286 one glycosylation site has been deleted (N to S). In this regard, increase in virulence that is correlated with additional glycosylation had not been detected (2,28,and 29). The present findings also indicate that the HA gene of the H9 influenza viruses circulating in Tehran province were not well conserved and in recent years had dominant changes. Especially from 2003, a dominant change had been produced in Iranian isolates. It is possible that these differences represent mutations that could have occurred during field passages. For further investigation, phylogenie study is essential. The results of this study did not identify the four Iranian AI isolates as highly pathogenic based on molecular-based pathogenecity detection. We assumed that co infections play a critical role in pathogenesis of AI viruses in Iran. Also changes in other genes of Iranian isolates might increase pathogenecity of these viruses in field. It is possible that changes in cleavage site are due to emergence of H9N2 from another source. In order to identify the source of the Iranian H9N2 outbreaks it is necessary to study the phylogenie study on nucleotide sequence of the HA gene or other genes of other Iranian H9N2 isolates from different sources including backyard flocks, waterfowl or migratory birds. Recently, H9N2 viruses have been isolated from migratory birds in Iran (30). Continuous surveillance would improve our understanding of the role of various avian hosts in ecology of influenza viruses and thus the underlying phenomena in emergence of pandemic strains. Also, according to H5Nl case report in non-industrial birds, it is very critical to detect any changes in H9N2 isolates. According to the findings of this study and emergence of new motif in Iran, the surveillance of internal genes for further investigation is very important.

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