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Original Article

Genetic Analysis of Hemagglutinin Protein of H9N2 Isolated from Live Bird Markets in Tehran Province in 2007-2008

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Abstract

Background and Aims: Influenza A virus subtype H9N2 have continued to circulate in domestic poultry farms in Asia since 1998. The virus circulates in live bird markets, missing link in epidemiology of avian influenza. Regarding previous studies on H9N2 viruses of Iran and having no data on this subject in Iranian live bird markets this study was conducted to analyze genetically hemagglutinin protein of H9N1 virus.

Methods: A total of 500 tracheal and cloacal swabs from clinically healthy birds of Tehran's live bird markets were collected. Diagnostic RT-PCR was done on them using specific primers for subtype H9N2. Eight positive samples were selected for inoculation into 9-11 days SPF emberyonated eggs and the virus was grown and isolated. Amplification of the HA gene was carried out by PCR using two pairs of specific primers. PCR products were separated, purified and cloned. The products were sequenced and analyzed with M13 primers. They shared high amino acid homology with genes of other H9N2 viruses isolated previously in Asia and Iran.

Results: H9N2 viruses isolated from live bird markets were highly similar to viruses isolated from industrial poultry being circulated as early as 2001.

Conclusion: The results suggest that a common source of H9N2 viruses is circulating in Iran.

Keywords: Avian influenza virus (H9N2); Live Bird Market; Hemagglutinin

Introduction

Avian influenza viruses (AIVs) belong to Influenza virus A genus of the Orthomyxoviridae family with negative strand segmented RNA genome. Influenza A viruses are divided in to subtypes on the basis of the position of one of 16 antigenically distinct Hemagglutinin (HA) antigens (H1-H16) and one of the nine NA (Neuraminidase) antigens (N1-N9). Cleavage of the HA to HA1 and HA2 is essential for infectivity and pathogenicity of AIV. The existence of multiple basic amino acids in HA1 and HA2 cleavage site is important for virus virulence especially H5 and H7 subtypes (1-3). Influenza A viruses infecting poultry are divided into 2 distinct groups on the basis of their ability to induce disease. The virulent viruses produce a severe disease which could result in flock mortality up to 100%. Low pathogenic avian influenza (LPAI) viruses can cause subclinical to mild respiratory disease which could become sever in complicated forms (4).

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Since 1998, H9N2 avian influenza viruses have caused important problems in Iranian poultry industry. H9N2 subtype has been enzootic in Asia (5-8). This subtype has induced mortality about 20-60% in its outbreaks in broiler chicken farms during 1998-2001 in Iran. H9N2 isolates which belong to Euro-Asia lineage are represented by A/Duck/Hong Kong/Y280/97 and A/Quail/HK/G1/97) groups, which the latest thought to be the donor of 6 internal genes to poultry and human H5N1 viruses isolated in 1997. For the first time, in Hong Kong and China in 1999 a case of human infection with subtype H9N2 of AIV was proved and showed these viruses could have potential to be transmited from birds to humans (9). It has been reported previously that H9N2 subtype may use receptor binding sites identical to viruses which induced influenza pandemic in human, so subtype H9N2 AIV viruses could be important to be considered for next pandemic (10-12).

Live bird markets thought to be a productive source and important reservoir for avian influenza viruses linked to outbreaks of influenza in industrial poultry and humans (12-14). Live bird markets provide a proper place for genetic mixing and spreading of influenza virus among different bird species. Low pathogenic avian influenza viruses have been isolated from live bird markets repeatedly. Existence of these viruses in the live bird markets could be dangerous for commercial poultry in different regions. Several genetic changes had occurred in hemagglutinin cleavage site that could result in an increase in virulence if the virus which continues to circulate in live bird markets (7, 11, 15-20). Regarding previous studies on H9N2 viruses of Iran and having no data on this subject in Iranian live bird markets this study was conducted to analyze genetically the hemagglutinin protein of H9N1 virus.

Methods

A total of 500 tracheal and cloacal swabs from clinically healthy birds of Tehran's live bird markets were collected. Sampling were taken from various species such as chickens (n=368), turkeys (n=102) and ducks (n=30). Up to 10 swabs from the same species within a market were pooled, into each transport media tube beside ice packs and shipped into laboratory. Viral RNA was extracted from tracheal and cloacal swabs using QIAamp viral RNA Mini kit (QIAGEN) according to the manufacturer's instruction. Reverse transcription was done by using universal primer Uni 12: 5'-AGC AAA AGC AGG-3' with revert aid "first strand cDNA synthesis kit (Fermentas, Canada). Amplification of the H9 and N2 genes were carried out by PCR using two pairs of diagnostic primers H9F: 5'- TTG CAC CAC ACA GAG AAT-3', H9R: 5'- TGA TGT ATG CCC CAC ATG AA-3', N2F: 5'- AGC AAA AGC AGG AGT GAA AAT GAA-3' & N2R: 5'- TTC TAA AAT TGC GAA AGC TTA TAT-3' (specific for H9 and N2) (Fig. 1 and Fig. 2) (21).

Diagnostic PCR for H9 and N2 was carried out using specific primers and the positive samples were selected. The positive samples were briefly vortexed, centrifuged and the supernatant was placed into Tris-buffer tryptose broth containing antibiotics. Specimen and antibiotic mixtures were inoculated into SPF 9-11 day old embryonated chicken eggs via the chorioallantoic sac rout. The eggs were inoculated at 36-37^{o}C for 4 days. The eggs were candled daily and the embryos which died within 24 hours post inoculation were discarded. The harvested allantoic fluids were assessed for presence of the virus by hemagglutination assay (22). Viral RNA was extracted from infected allantoic fluid using RNX reagent according to the manufacturer's instruction (Cinnagen, Iran). The RNAs of isolates were reversing transcripted by using oligonucleotide influenza universal primer Uni 12: 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 using two pairs of specific primers. These primers were used for complete and simple extraction of HA gene, these two pairs of primers were overlapped together from nucleotide number 766 to 832, so the first primer amplified nucleotide number 1 to 832 F1:5'-AGC AAA AGC AGG GG-3' & R1: 5'-

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GTG YCC ATA CCA TGG RGC-3' and the second one amplified nucleotide number F2: 5'- ACC AGG TCA GAC ATT GCG AG-3' and R2: 5'- AGT AGA AAC AAG GGT GTT TTT GC-3' 766-1742 (22).

The PCR products were assessed by gel electrophoresis (Fig. 3 and Fig. 4). PCR product purified by DNA Extraction Kit (Fermentas-Canada) from agarose gels according to the manufacturer's instruction. This work was done for all 8 isolates which were selected by diagnostic PCR in the first step. After purification the products were cloned with *Ins* T/A cloning kit (Fermentas-Canada) according to the manufacturers instruction. Plasmid extraction was carried out by Plasmid isolation kit (Fermantas-Canada). The products were sequenced and analyzed with M13 primers by plasmid sequencing on an automated sequencer (Cinnagen-Iran). Nucleotide and amino acid sequences were edited by Editseq™ program. Amino acid sequences were done by Generunner on the basis of nucleotide sequence. Sequences were aligned with clustalW (laser gene DNA star Madison WI) and Megalign™. Sequence data were analyzed using sequence analysis software package (DNASTAR) (23). The nucleotide sequences of H9N2 influenza viruses used in this study obtained from gene

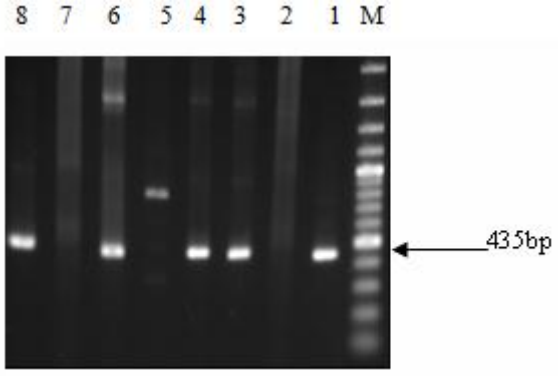

Fig. 1. Diagnostic Gel electrophoresis of the H9 (435bp) RT-PCR products of the H9N2 viruses isolated from live bird markets of Tehran province during 2007**.** M= ladder 100bp (Fermentas), lane 1= (H9 subtype) positive control, lane 2= negative control, lanes 3, 4, 6 and 8= samples contain H9 subtype, lanes 5 and 7= samples do not contain H9 subtype.

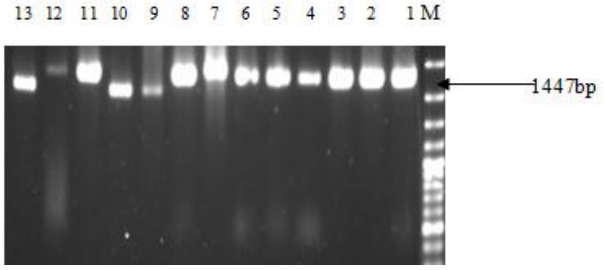

Fig. 2. Diagnostic Gel electrophoresis of the H9 (1447bp) RT-PCR products of the H9N2 viruses isolated from live bird markets of Tehran province during 2007**.** M= ladder 100bp (Fermentas), lane 1= (N2 subtype) positive control, lane 2, 3, 4, 5, 6, 8 and 13 = samples contain N2 subtype, lanes 7, 9, 10, 11 and 12= samples don't contain H9 subtype.

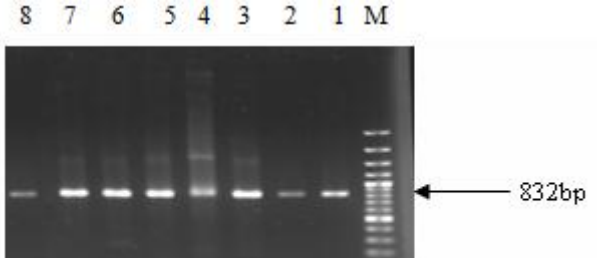

Fig. 3. First strand PCR product (832bp) eight virus isolates RT-PCR products of the H9N2 viruses isolated from live bird markets of Tehran province during 2007. M= ladder 100bp (Fermentas), lane 1= THLBM861, lane 2= THLBM862, lanes 3= THLBM863, lanes 4= THLBM864, lanes 5= THLBM865, lanes 6= THLBM866, lanes 7= THLBM867, lanes 8= THLBM868.

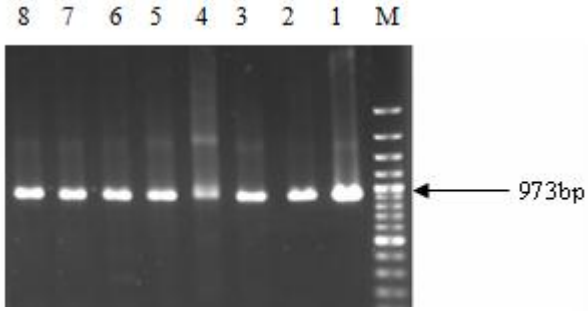

Fig. 4. Second strand PCR product (973bp) eight virus isolates subtype H9N2 from live bird markets of Tehran province in 2007. M= ladder 100bp (Fermentas), lane 1= THLBM861, lane 2= THLBM862, lanes 3= THLBM863, lanes 4= THLBM864, lanes 5= THLBM865, lanes 6= THLBM866, lanes 7= THLBM867, lanes 8= THLBM868.

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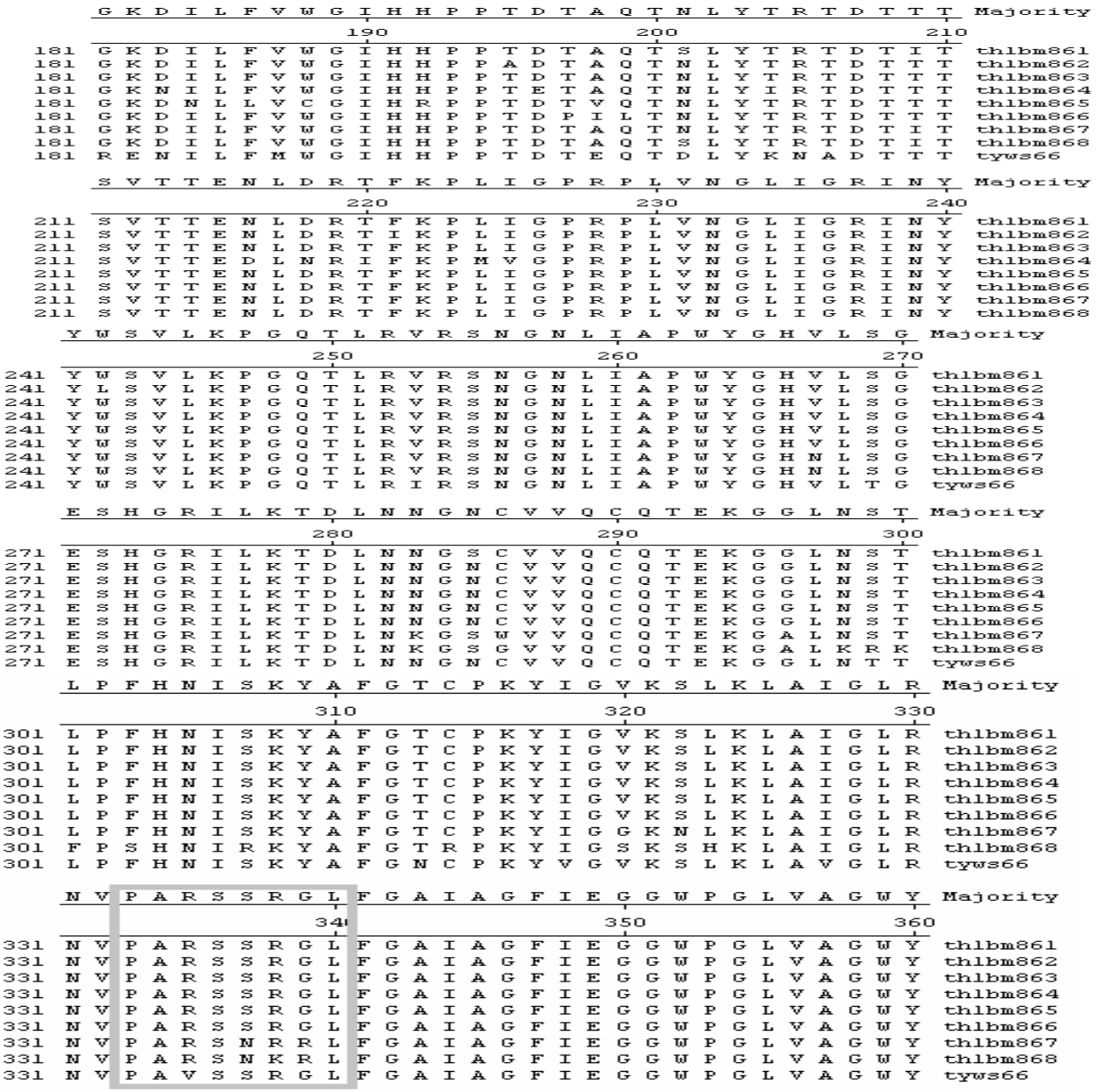

Fig. 5. Amino acid analysis of a part (180-360) of the H9 of the H9N2 Gray rectangle: cleavage site (332-340)

*Amino acid position is numbered according to the turkey/Wisconsin/66 (*DNASTAR, Megalign*^{TM}).

bank under accession numbers GUO71977 through GUO71984 (Table 1).

Results

Eight H9N2 subtypes were selected by specific primers for H9 and N2. After isolation of viruses, RT-PCR, cloning and sequencing isolates had 1732-1737 nucleotides. The most amino acid homology was observed between isolates THLBM861 and THLBM867 (97.1%) (Table 2). The isolates showed high similarity with Iranian isolates (ICIEU477244& efo63731 & efo63733 & efo63734) (Fig. 6).Amino acid sequence (180-360) of HA were deduced from the nucleotide sequence (Fig. 5). Eight new Iranian isolates were compared with other published Iranian isolates (Fig. 6). Amino acid sequence at the cleavage site of the HA of the isolates possessed –P-A-R-S-S-R/G-

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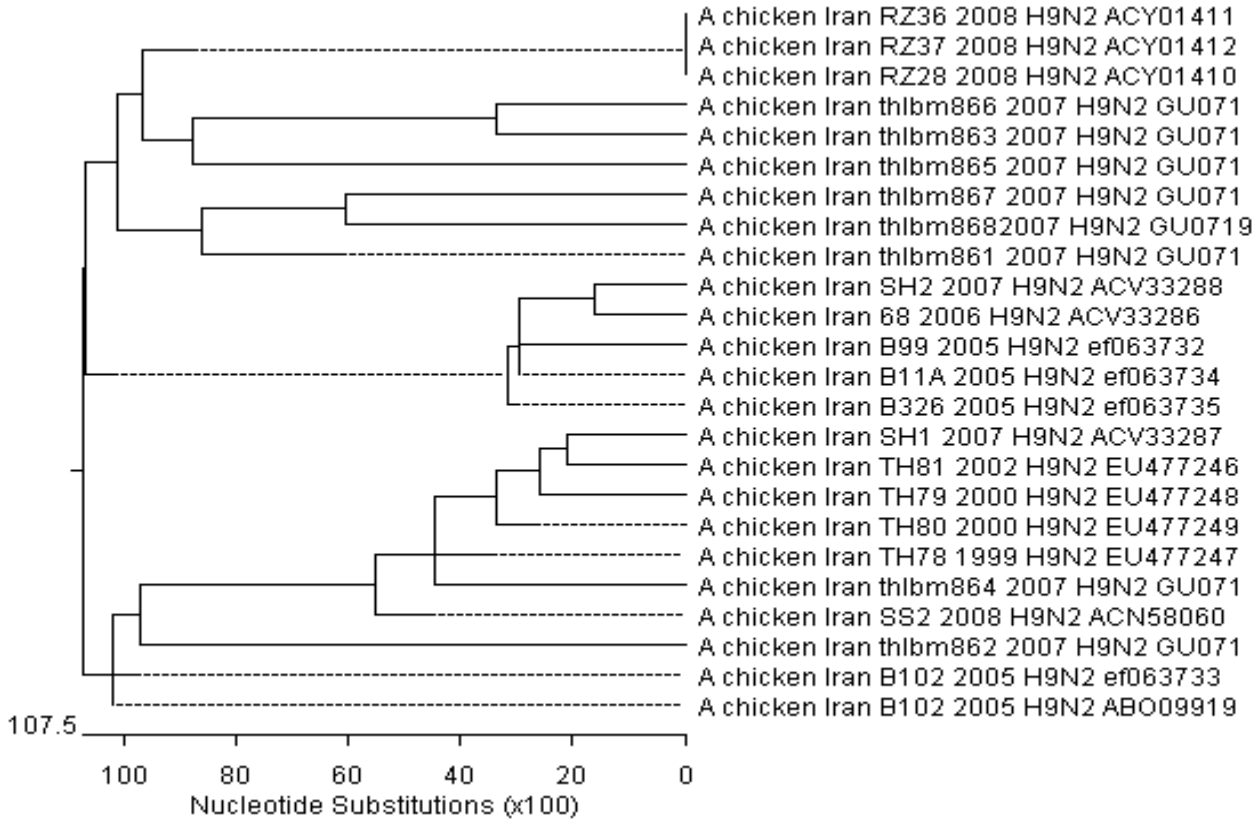

Fig. 6. Phylogenetic tree for the H9N2 HA gene of influenza viruses of Iran. Amino acids 1-560 of HA were used for the phylogenetic analyses. Horizontal distances are proportional to the minimum number of amino acid differences required to join nodes and sequences. (*DNASTAR, Megalign*^{TM}).

L motif, except 7th Isolates(S TO N and G to R) and 8th isolate (S to N, R to K& G to R). Amino acid changes in these two isolates were new (Fig. 5).

Discussion

Recently, avian influenza viruses subtype H9N2 induced major economic losses in Iranian poultry industry. Besides existing vast amount of data for type A influenza in industrial host species, there is limited information from live bird markets about H9N2 subtype in the world and specially there is no data about this subtype of avian influenza in live bird markets of Iran. Live bird markets as already mentioned are believed to be one of the best environments for keeping avian

Table 1. Sequences pair distances of Q.MEG. Using Clustal method with PAM250 residue weight table.

1	THLBM861	A/Chicken/Iran/ THLBM861 /2007 (H9N2)	GUO71977
2	THLBM862	A/Chicken/Iran/ THLBM862 /2007 (H9N2)	GUO71978
3	THLBM863	A/Chicken/Iran/ THLBM863/2007 (H9N2)	GUO71979
4	THLBM864	A/Chicken/Iran/ THLBM864 /2007 (H9N2)	GUO71980
5	THLBM865	A/Chicken/Iran/ THLBM865/2007 (H9N2)	GUO71981
6	THLBM866	A/Chicken/Iran/ THLBM866 /2007(H9N2)	GUO71982
7	THLBM867	A/Chicken/Iran /THLBM867 /2007(H9N2)	GUO71983
8	THLBM868	A/Chicken/Iran/ THLBM868 /2007(H9N2)	GUO71984

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Table 2. divergence level of eight AIVs isolated in this study (*DNASTAR, Megalign*^{TM}).

Percent Identity

Divergence

	1	2	3	4	5	6	7	8	
1		93.2	97.0	94.1	93.6	96.4	97.1	96.8	1 thlbm861
2	7.1		93.0	92.1	90.0	92.3	91.2	90.4	2 thlbm862
3	3.1	7.3		94.3	93.9	97.1	95.0	94.1	3 thlbm863
4	6.1	8.3	6.0		90.9	93.4	92.3	91.2	4 thlbm864
5	6.7	10.8	6.3	9.7		93.9	91.8	90.7	5 thlbm865
6	3.7	8.1	2.9	6.9	6.3		94.5	93.6	6 thlbm866
7	2.9	9.3	5.2	8.1	8.7	5.8		96.4	7 thlbm867
8	3.3	10.3	6.1	9.3	9.9	6.7	3.7		8 thlbm868
	1	2	3	4	5	6	7	8	

influenza viruses and transmission between species and productive source for mixing and reassortment of new viruses with higher pathogenicity (24). They are in close contact with human and with each other and can transfer viruses to human. 500 samples were taken and 8 influenza viruses of H9N2 subtypes isolated. All of our isolates were from chickens probably because of the frequency of chicken samples comparing to other species.

Antigenic and phylogenetic analysis showed that all 8 H9N2 isolates were of Eurasia lineage similar to lineage caused disease in chickens in Iran but were different from H9N2 viruses of North America (25-28).

Genetic analysis revealed that they were in similar phylogenetic groups' isolated in previous studies of industrial poultry in 2007 based on H9 subtype HA gene sequences (22). Deduced amino acids of the AIV isolates were not highly pathogenic on molecular basis, but there were some substitutions in some of nucleotides. Little information for the HA of H9N2 subtype is available from live bird markets to draw conclusion. Hemagglutinin (HA) genes were sequenced and compared phylogenetically with available isolates. Sequence analysis of the HA gene of these 8 isolates showed that viruses were most similar to previous studies in 2007 industrial poultry of Iran (22), which could suggest a common source for H9N2 viruses circulating in Iran, indicating that these isolates probably have been evolved from a common source (Fig. 6).

Nucleotide sequences around the cleavage site was (SR-GL) for the first 6 isolates, (NR-RL) for 7th isolate, and (NK-RL) for 8th isolate which were indicative of low pathogenic avian influenza viruses. These isolates show some levels of substitution, indicating antigenic drift. In this study three different sequences exist in cleavage site position that 6 isolate have the –P-A-R-S-S-R/G-L motif which is similar to the previous ones isolated in Iran (22) but the 7th and 8th isolate have new motifs with addition of a basic amino acid, which could propose the oncoming danger for converting these viruses to highly pathogenic avian influenza viruses if other necessary changes happen The cleavage site sequence is not similar to highly pathogenic viruses' motifs yet (R-X-R/K-R). It is the first report for the emergence of new

Table 3. Comparison of amino acid positions on hemagglutinin protein receptor binding site on the basis of H3.

Isolates	98 (106)	153 (161)	155 (163)	183 (191)	190 (198)	194 (202)	195 (203)	226 (234)
H3	Y	W	T	N	A	L	Y	L
THLBM861	G	W	T	H	A	L	Y	L
THLBM862	G	W	T	H	A	L	Y	L
THLBM863	G	W	T	H	A	L	Y	L
THLBM864	G	W	T	H	A	L	Y	L
THLBM865	G	W	T	H	V	L	Y	L
THLBM866	G	W	T	H	I	L	Y	L
THLBM867	G	W	T	H	A	L	Y	L
THLBM868	G	W	T	H	A	L	Y	L

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Table 4. Glycosylation sites existed in this study (NXT/S motif, X: any amino acid except prolin).

	Amino acid position	Glycosylation sites	Detected	Deleted
1	29-32	NSTE	All isolates	
2	105-108	NGTC	All isolates	
3	141-144	NVTY	All isolates except THLBM865	THLBM865
4	298-301	NISK	All isolates except THLBM868	THLBM868
5	305-308	NISK	All isolates	
6	492-495	NGTY	All isolates	
7	551-554	NGSC	THLBM861, THLBM862, THLBM864, THLBM865, THLBM868	THLBM863
		NGTC	THLBM866, THLBM867	

substitution of amino acid in hemagglutinin cleavage site of Iran live bird markets. The isolate may have acquired mutations in the HA gene during the transmission in live bird markets. The complete coding sequence included nucleotides 1732-1737, which encoded for a protein of 560 amino acids. The glycosylation sites were 7 places which 5 of them were in HA1 and 2 places were in HA2. They were related to HA protein functions but were not completely conserved and almost similar to previous studies in Iranian isolates with some differences which seems unimportant. It is possible that these differences represent mutations that could have occurred in markets, there were no additional glycosylation site could induce virus virulence (Table 4) ([23]). This study isolates all have leucin amino acid in 234 positions and so have the potential for binding to human receptors (Table 3). Receptor binding sites of the isolates in this study are somewhat similar to human isolate (H3) which could be a danger (Table3). The left edge of binding pocket in all of this study isolates had the NGLIGR motif which is similar to human isolates and the previous isolates of Iran. So on the basis of this study hypothesis some changes had occurred in live bird markets of Tehran province, These changes in cleavage site may be due to emergence of new H9N2 viruses from different sources (different markets, countries species, and etc). Continuous surveillance would improve our understanding of the real role of live bird markets of Iran in ecology of influenza viruses and thus the underlying phenomena in emergence of pandemic strains.

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