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Nucleotide and Amino Acid Sequence Analysis of Hemagglutinin Protein in Cleavage Site Region of H9N2 Isolated from Broilers in Tehran Province during 1998-2007

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Abstract: Iranian poultry industry has been affected by Avian Influenza (AI) virus Since 1998, subtype H9N2. The association of high mortality and case report of H5N1 and H9N2 influenza virus in wild birds in recent years raised the specter of a possible new genetic modified AI virus. Partial nucleotide sequences and deduced amino acid of Hemagglutinin (HA) genes in cleavage site region of 9 H9N2 influenza viruses isolated from broilers in Tehran Province during 1998-2007 were genetically analyzed. The isolates possessed two types of amino acid motif –R–S–S–R/G–L– and –R–S–N–R/G–L - at the cleavage site of HA. "–R–S–N–R/G–L in Iranian isolates is the same as the motif previously reported in Israel. The results of this study indicated that all of Iranian viruses have potential for emerging for highly pathogen influenza virus, thus continuously monitoring of viruses is essential in Iran.

Key words: H9N2, chicken, hemagglutinin, cleavage site, Iran

INTRODUCTION

Avian influenza has emerged as a disease with significant potential to disrupt commercial poultry production often resulting in extensive losses (Alexander, 2000). Influenza is caused by a zoonotic virus that occurs in lower animals and birds as well as in humans). Influenza viruses belong to the Orthomyxoviridae family of RNA viruses and are divided into five genera: Influenza A, B and C virus, Thogtovirus and Isavirus. Avian Influenza (AI) is a highly contagious disease caused by type A influenza viruses, which have segmented negative-strand genomes (Lamb and Krug, 2001; Swayne and Halvorson, 2003). The Hemagglutinin (H) and Neuraminidase (N) are two structurally distinct envelope glycoproteins attached to the lipid bilayer to form the surface of the virion. HA is the major antigen for neutralizing antibodies and is involved in the binding of virus particles to receptors on host cells (Lamb and Krug, 2001; Swayne and Halvorson, 2003). The antigenic differences of hemagglutinin and neuraminidase antigens of influenza viruses provide the basis of their classification into subtypes. Serologically there are 16 H and 9N subtype of AI virus antibodies in chicken (Lamb and Krug, 2001; Fouchier *et al*., 2005). Avian Influenza (AI) outbreaks in commercial chickens and turkeys are characterized by the sign of respiratory distress, decrease egg production and mortality rates that ranges up to almost 100%. Field evidence shows that a variety of pathogenicity differences poorly defined host and environmental factors also play an important role in determining the outcome of infection (Swayne Halvorson, 2003). Since 1998, H9N2 AI outbreaks have been one of the major problems in Iranian poultry industry (Nili and Asasi, 2003; Vasfi Marandi and Bozorgmehrifard, 1999; Pourbakhsh *et al*., 2000). Last H9N2 influenza virus outbreak in broilers in chicken farms during 1998-2001 in Iran, the mortality rate was 20-60% (Nili and Asasi, 2002).

H9N2 isolates are represented by three groups: A/Duck/HongKong/Y280/97 (A/Dk/HK/Y280/97) and A/Quail/Hong Kong/G1/97 (A/Qa/HK/G1/97) (Banks *et al*., 2000). A/Qa/HK/G1/97 is thought to be the donor of 6 “internal” genes to the poultry and human H5N1 viruses isolated in 1997 (Guan *et al*., 1999). Documented cases of human infection with H9N2 avian influenza viruses, first detected in 1999 in Hong Kong and China, indicate that these viruses can be directly transmitted from birds to humans. Previous studies have documented the ability of H9N2 viruses to acquire receptor and binding characteristics that are similar of the human pandemic influenza viruses. Thus H9N2 influenza viruses are considered to be one of candidates for the next pandemic (Peiris *et al*., 1999a; Butt *et al*., 2005).

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Table 1: Cleavage site motif variations amongst H9 influenza viruses in the world (19)

Variant	Cleavage site motif	Motif	Virus strain	Gen bank accession number
1	ALDR	-	A/duck/Hong Kong/366/78	AY206674
2	ASAR	L	A/turkey/Germany/R33/96	AJ781821
3	ASDR	C	A/duck/Hong Kong/784/79	AF156383
4	ASGR	B	A/duck/Hong Kong/168/77	AF156382
5	ASNR	J	A/duck/Hong Kong/147/77	AY206671
6	ASYR	-	A/ostrich/South Africa/9508103/95	AF508554
7	GSSR	M	A/chicken/Guangdong/SS/94	AF384557
8	ISGR	H	A/turkey/Italy/125/89	AF218095
9	ISNR	N	A/pekin duck/Singapore/F91-5/9/97	AF218105
10	KSSR	I	A/chicken/Jiangsu/2/98	AF461510
11	RLSR	-	A/sw/ShanDong/1/2003	AY294658
12	RSKR	-	A/turkey/Givat Haim/868/02b	DQ104471
13	RSNR	-	A/chicken/Sede Uzziyyahu/1651/04b	DQ108923
14	RSSR	K	A/Hong Kong/1073/99b	AB080226
		O	A/avian/China/417-428/98	AF218106
15	TSGR	E	A/turkey/Italy/VR86/83	AF218088
16	TSNR	F	A/turkey/Itali/245/84	AF218106
17	VSDR	D	A/duck/Hong Kong/448/78	AB080224
18	VSNR	G	A/duck/Hong Kong/702/79	AY206672
19	VSSR	A	A/duck/India/31g/86	AF218091

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 intracellular as an inactive protein-precursor (HA0). In the course of the infection, HA0 is cleaved into two disulfide-linked polypeptide chains, HA1 and HA2 (Klenk and Garten, 1994). The cleavage site of 13 from 16 HA subtypes (all HA subtypes but H5 and H7) consist of 4 amino acids including a single arginine residue which is removed from the COOH terminus of the HA1 after cleavage by means of virus-associated carboxypeptidase (Garten and Lenk, 1983). A correlation between cleavability of the HA0 and pathogenicity has been established. The HA0 protein of non-pathogenic influenza viruses can be cleaved extra cellular by trypsin-like enzymes originating from co-infecting bacteria or host inflammatory response induced by respiratory tract infections. 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 extra cellular serine proteases. That recognize Q/E–X–R motif. In a few cases of the H5 and H7 subtypes, in which polybasic sequences are inserted between HA1 and HA2 chains, the cleavage is carried out by the intercellular subtilisin-like enzymes, which recognize R–X–R/K–R motif. However, R–X–R/K–R motif presence in cleavage site is not sufficient for successful cleavage; it has been shown that the cleavage site of the majority of influenza viruses (consisting of 4 amino acid) is inaccessible for the action of intracellular furin-like proteases. A cleavage site becomes accessible for intracellular proteases after either insertion of at least 2 additional basic amino acid, or after removing lateral carbohydrate chain (Bosch *et al*., 1981; Webster and Rott, 1987; Horimoto *et al*., 1995; Ohuchi *et al*., 1989). The cleavage site amino acid sequence is specific in each region. All type of this motif has been show in Table 1 (Perk *et al*., 2006). The association of high mortality in recent years and report of H5N1 and H9N2 in wild birds in Iran (WHO, 2008) raised probability of a possible new genetic modified AI virus. For this reason that the most molecular research on Iranian H9N2 isolates in Cleavage site, In this study, we analyzed nucleic acid as well as amino acid sequence of the cleavage site of hemagglutinin protein of H9N2 from Iranian isolates from poultry in Tehran, Iran in during 1998-2007.

MATERIALS AND METHODS

Sample collection: Sample collection was performed according to the standard method from suspected clinical broiler specimens in Tehran Province in 2007. Specimens (lung and intestine) were stored at -70°C until use. Samples were collected in a 2X phosphate buffer solution (PBS, pH 7.4) containing antibiotics and anti antifungal (SIGMA, St. Louis, MO, USA). Other virus samples are available in central lab of department of clinical science in faculty of veterinary medicine (University of Tehran).

Virus isolation: Ten-day-old embryonated chicken eggs were inoculated and incubated at 37°C for 48 h. Eggs were candled daily and embryos dying within 24 h post inoculation were discarded. Allantoic fluids were collected from the eggs and the presence of viruses was determined by hemagglutination assay. The identification of viruse subtype was determined by a standard Hemagglutination Inhibition (HI) and Neuraminidase Inhibition (NI) tests

using polyclonal chicken antisera. The allantoic fluids containing virus were harvested and stored at -70°C until use. All of HA negative Alantoic fluids were inoculated for the second passage (WHO, 2002).

RNA extraction: Viral RNA was extracted from infected allantoic fluid using RNX reagent according to the manufacturer's instruction. Briefly, in an RNAase free 1.5 mL tube, 800 μL of RNX TM-Plus solution (Cinnagen, Iran) was added to 200 μL allantoic fluids. After shaking, 200 μL of chloroform was added and the mixture was centrifuged at 14,000 g at 4°C for 15 min. Equal volume of Isopropanol was added to the upper phase in a new tube. The mixture was centrifuged at 12,000 g at 4°C for 15 min. The supernatant was discarded and 500 μL of 75% ethanol was added to the pellet. After centrifugation at 7,500 g for 10 min 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 μL DEPC water. To help dissolving, place the tube in 55-60°C water bath for 10 min and store at -70°C for RT-PCR reaction (Peiris *et al*., 1999b).

RT and PCR reaction: Reverse transcription was done by using oligonucleotide influenza universal primer uni12: 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 by using one pairs of specific primers. Primer sequences are available upon request. The reaction mixture (50 μL) contained 5 μL of cDNA, 15 pmoles of forward and reverse primers (4 μL) and Cinnagen master mix (Cinnagen, Iran). The amplification protocol was: One step of denaturation at 94°C for 3 mins, 35 cycles of 94°C /45 Sec, 58°C /45 Secs and 72°C/60 Secs and final extension at 72°C for 10 min. 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 and USA) (Hoffmann *et al*., 2001).

TA cloning and sequencing: Purified PCR products for sequencing were cloned into plasmid for TA cloning with Ins T/A cloning (Fermentas, Canada) according to the manufacturer's instruction. Plasmid extraction from positive clone was carried out by QIA miniperep plasmid extraction kit (Qiagen, Valencia, CA, USA). Following digestion with ECORI (Fermentas, Canada) to confirm the insertion, the nucleotide sequences were analyzed by plasmid sequencing on an automated 3700 DNA sequencer (Applied Biosystems, Foster city, CA). The sequences were resolved using the ABI RIS Mcollection program (Perkin-Elmer, Foster City, CA) with M13 (Forward and Reverse) universal primer.

Table 2: Isolates characterized in this study

No	Access no.	Virus name	Virus symbol
1	EU477241	A/Chicken/Iran/TH77/1998(H9N2)	TH77
2	EU477247	A/Chicken/Iran/TH78/1999(H9N2)	TH78
3	EU477248	A/Chicken/Iran/TH79/2000(H9N2)	TH79
4	EU477249	A/Chicken/Iran/TH80/2001(H9N2)	TH8
5	EU477246	A/Chicken/Iran/TH81/2002(H9N2)	TH81
6	EU477242	A/Chicken/Iran/TH85/2007(H9N2)	TH85
7	EU477245	A/Chicken/Iran/TH186/2007(H9N2)	TH186
8	EU477243	A/Chicken/Iran/TH286/2007(H9N2)	TH286
9	EU477244	A/Chicken/Iran/TH386/2007(H9N2)	TH386

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

Nucleotide sequence accession numbers: The nucleotide sequences for all H9N2 influenza viruses used in this study are available GenBank under accession numbers EU477241 through EU477249. The accession numbers are provided in Table 2.

RESULTS AND DISCUSSION

In recent years, AI has caused major economic harms in Iranian poultry industry. The latest Iranian H9N2 isolate has been reported to be low pathogenic for SPF chickens. However, recent H9N2 outbreaks have caused

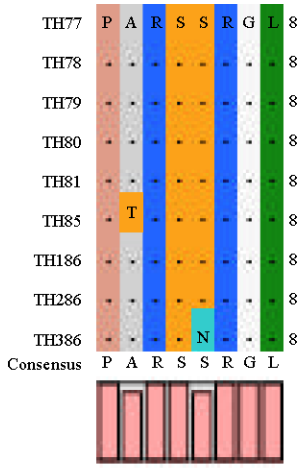

Fig. 1: Comparison of amino acid sequence of H9N2 iranian isolates in cleavage site region

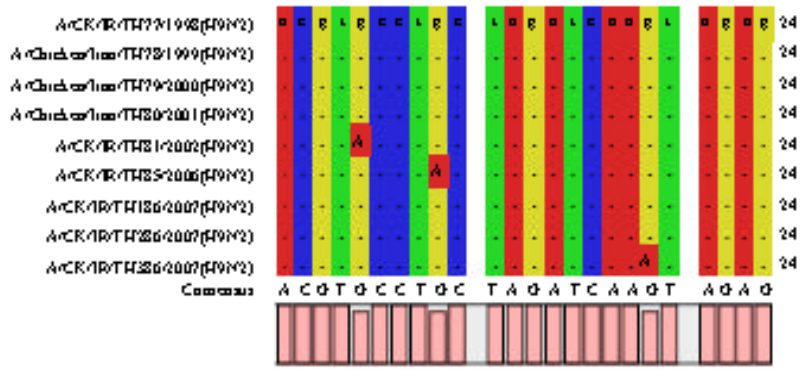

Fig. 2: List of the H9N2 viruses Nucleotide sequences at cleavage site region in this study

up to 65% mortality rate and are the only influenza subtype in the chicken population in Iran (Nili *et al*., 2003; Vasfi Marandi *et al*., 1999; Pourbakhsh *et al*., 2000). Although, pathogenicity of avian influenza virus is a multifactor the amino acid sequence of cleavage site of HA is considered to be its major determinant. The pathogenicity 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 A/H9N2 subtype virus is related to pathogenicity (Fig. 1), although it is similar to the motif (R-X-R/K-R) required for highly pathogenic viruses of H5 and H7. Only one nucleotide substitution could change the -R-S-S-R- motif to -R-S-R-R- and it was previously (Bosch *et al*., 1998; Webster *et al*., 1987; Horimoto *et al*., 1995; Ohuchi *et al*., 1989) suggested that H9N2 viruses prevalent in Iran are potentially capable of becoming highly pathogen. The presence of Serine amino acid has made it a crucial motif because only 1 nucleotide substitution (C to A or G) at 2 positions is enough to convert this motif to virulent sequence. To determine the epidemiological characteristics and the HA cleavage site motifs of the 9 Iranian H9N2 isolates, the corresponding PCR products comprising the HA cleavage site were studied and compared to those from other H9N2 isolates.

The amino acid sequence in cleavage site sequence of HA were deduced from the nucleotide sequence. Amino acid sequences at the cleavage site of the HA of the isolates possessed -P-A-R-S-S-R/G-L- motif, except two isolated: TH85 (A to T) and TH386 (S to N). All changes in region of Nucleotide sequence that coding cleavage site is available in Fig. 2.

None of the 9 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 (Peiris *et al*., 1999b). This motif doesn't exist in Iranian isolation before 2007. Amino acid in cleavage site of TH85(PTRSSRG) is found in isolates with accession number AAL65235 and ABV46459. These isolates are in Chinese isolates group. During 10 years of observation, –R–S–S–R–, motif predominated amongst the Iranian isolates (Toroghi *et al*., 2003; Kianizadeh *et al*., 2006; Torokhi *et al*., 2006; Karimi *et al*., 2004), new motifs were 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 nucleotide substitutions in the HA-connecting peptide region. About some positions differences in this study isolates, these changes in amino acid sequence had not been detected in Iranian isolates before 2004.

CONCLUSION

The present findings also indicate that the HA genes of the H9 influenza virus circulating in Tehran province were not also well conserved and in recent years have 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. Particularly, TH 386 probably originated from Israel. For further investigation, phylogenetic study is essential. The results of this study did not identify the nine Iranian AI isolates as highly pathogenic as 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 to increase pathogenicity of these viruses in field. It is possible changes in cleavage site are due to iterance of H9N2 from

another source. In order to identify the source of the Iranian AI H9N2 outbreaks it is necessary to study the phylogenetic 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, AI H9N2 viruses have been isolated from migratory birds in Iran (Fereidouni *et al*., 2005). 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 H5N1 case report in non industry birds, it is very critical for H9N2 monitoring for probably changes in H9N2 isolates. According to this reason and emergence of new motif in Iran, it is very critical for surveillance internal genes for further investigation.

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