Genetic and phylogenetic analysis of the ribonucleoprotein complex genes of H9N2 avian influenza viruses isolated from commercial poultry in Iran

Bashashati, M.[1], Vasfi Marandi, M.[1*], Bozorgmehri Fard, M.H.[1], Hemmatzadeh, F.[2], Sabouri, F.[1]

[1]*Department of Poultry Diseases, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran*

[2]*School of Animal and Veterinary Sciences, University of Adelaide, Adelaide, South Australia, Australia*

Key words:
avian influenza virus, H9N2, poultry, ribonucleoprotein

Correspondence
Vasfi Marandi, M.
Department of Poultry Diseases,
Faculty of Veterinary Medicine,
University of Tehran, Tehran, Iran
Tel: +98(21) 66923510
Fax: +98(21) 66933222
Email: mvmarand@ut.ac.ir

Received: 8 January 2013
Accepted: 5 May 2013

Abstract:

BACKGROUND: The H9N2 subtype of avian influenza viruses (AIVs) has been isolated in multiple avian species in many European, Asian, African and American countries. Since the first outbreak of H9N2 virus in Iran in 1998, this virus has widely circulated throughout the country, resulting in major economic losses in chicken flocks. Several amino acids in the virus ribonucleoprotein (RNP) complex including the nucleoprotein (NP) and polymerase (PB2, PB1 and PA) proteins are associated with host range and virulence. **OBJECTIVES:** Our aim was to understand the molecular characterization of RNP complex proteins of Iranian H9N2 subtype isolates. **METHODS:** The full length nucleotide sequences of RNP complex genes of two strains designated as Ck/IR/ZMT-101/98 and Ck/IR/EBGV-88/10 were amplified and sequenced. **RESULTS:** The phylogenetic analysis revealed that both strains were located in different sub-lineages. However, based on the genetic similarities, PB1, PA and NP genes of Ck/IR/EBGV-88/10 strain had a close relationship with a H7N3 subtype strain, isolated from Pakistan. Most positions of RNP proteins contained amino acids typical of avian determinants of host range. The results showed that the Iranian RNP complex genes have undergone genetic reassortment. **CONCLUSIONS:** Continuous AIV monitoring in poultry industry would help to obtain more information about genetic variation of H9N2 viruses and possible emergence of virulent and/or pandemic viruses.

Introduction

After the first description of H9N2 subtype of avian influenza virus (AIV) from turkeys in Wisconsin in 1966, infection of poultry with this subtype widely occurred in many countries in the mid-1990s. Since 1998, there have been reports of H9N2 infections in the Middle East and Asia, often causing widespread outbreaks in commercial chickens. Subsequently, vaccination was used to bring the disease under control in several countries (Naeem et al., 1999; Vasfi Marandi and Bozorgmehri Fard, 2002). However, H9N2 infection has become endemic among commercial poultry in numerous countries (Alexander, 2007).

In 1999, H9N2 infection of humans was reported from two children with mild upper respiratory tracts infections in Hong Kong. These two isolates are very closely related to a H9N2 virus isolated from a quail in Hong Kong. Genetic analysis revealed that the internal genes of these viruses were similar to those of human and avian H5N1 subtype isolated in 1997 (Lin et al., 2000). Furthermore, in a previous study, it has been shown that H9N2 viruses from Hong Kong

live bird markets have receptor specificity similar to that of human H3N2 viruses (Matrosovich et al., 2001). These findings highlight the public health concern and the necessity for more comprehensive surveillance of H9N2 subtype viruses among poultry and human populations.

The ribonucleoprotein (RNP) complex consists of nucleoprotein (NP), polymerase (PB2, PB1 and PA) proteins and viral RNAs. The RNP complex genes are known to play a role in the pathogenicity of influenza virus and harbor host-associated genetic signatures. Several mutations in these genes support better replication of AIVs in mammalian hosts (Chen et al., 2006; Katz et al., 2000; Manzoor et al., 2009; Naffakh et al., 2008; Shaw et al., 2002; Taubenberger et al., 2005). Therefore, it is important to monitor mutations in the genes of RNP complex to detect mutations associated with virulence, host range and interspecies transmission.

Since the first isolation of H9N2 virus in Iran in 1998, infection has become widespread in commercial poultry industry of the country. Although this virus is classified as low pathogenic AIV, it has caused severe mortality rate of 65% in some broiler farms as a result of co-infection with other respiratory pathogens (Vasfi Marandi and Bozorgmehri Fard, 2002). Despite the extensive use of vaccination against the disease (Vasfi Marandi et al., 2002), severe outbreaks have continued to occur in vaccinated flocks. Hence, it is important to know whether the H9N2 circulating virus has undergone further significant genetic evolution despite the application of this control policy. Most of the domestic studies have focused on the surface glycoproteins since they undergo frequent antigenic variation and play a crucial role in the virulence of AIVs. In contrast, there has been limited information about the internal genes of these viruses. To our knowledge, the data of molecular characterization and phylogenetic analysis of RNP complex genes of H9N2 viruses from Iran is not fully reported. Sequence analysis and phylogenetic study were carried out by comparing the four segments related to RNP complex genes of H9N2 with those of H9N2 viruses obtained from GenBank.

Materials and Methods

Virus Isolation: Two Iranian H9N2 AIVs were used in this study, Ck/IR/ZMT-101/98 as a reference virus and Ck/IR/EBGV-88/10 which was isolated from broiler chickens in H9N2 vaccinated poultry flocks in Tehran province in February 2010. The broiler chickens were 35 days old and they showed severe respiratory symptoms with 45% rate of mortality. Virus isolation was performed in allantoic cavity of 10 days old embryonated chicken eggs (ECE). Confirmation of Haemagglutination (HA) and Neuraminidase (NA) subtypes were conducted by two sets of specific primers for H9 and N2 in RT-PCR (Lee et al., 2001; Qiu et al., 2009). Infectious allantoic fluids were harvested from ECEs and kept in -70 for further use.

RNA Extraction and RT-PCR: Viral RNA was extracted from infectious allantoic fluids using RNX™-plus solution (Cinnagen, Iran) according to the manufacturer's instructions. The RNA was reverse transcribed by RevertAid™ first strand cDNA synthesis kit (Fermentas, Canada) and Uni12 primer, 5`-AGCAAAAGCAGG-3` (Hoffmann et al., 2001). PCR was carried out using Pfu DNA polymerase (Fermentas, Canada) and universal primers of influenza virus (Hoffmann et al., 2001).

Gene Sequencing and Phylogenetic Analysis: PCR products were purified with the GeneJET™ gel extraction kit (Fermentas, Canada) and subsequently cloned into ClonJET™ PCR cloning kit (Fermentas, Canada). Sequencing was performed by a DNA service company (Source BioScience Co., UK) in both directions. Internal primers were designed for each of the polymerase genes (primer sequences are available on request).

The sequences were compiled and aligned using BioEdit Package, version7.1.3.0. The Blast analysis was used to retrieve the homologous sequences for the analyzed sequences. Unrooted phylogenetic trees were constructed by the neighbor joining method using MEGA software, version 5.0 (Tamura et al., 2011). The robustness of the trees was evaluated by 1000 bootstrap replicates.

Accession Numbers of Nucleotide Sequences: The eight full-length nucleotide sequences for RNP complex including PB2, PB1, PA and NP genes of both Iranian isolates were deposited in GenBank under accession numbers: JX465619, JX465620, JX465621, JX465622, JX465623, JX465624, JX465627 and JX465628.

Table 1. Nucleotide sequence identity of RNP complex genes between Iranian isolates and representative viruses. [(a)] Abbreviations: Aq, aquatic; Ck, chicken; DK, duck; Qa, quail; Pa, Parakeet; Ty, Turkey; AE, Arabian Emirates; BJ, Beijing; CH, Chiba; DU, Dubai; EG, Egypt; GD, Guangdong; HK, Hong Kong; IL, Israel; IN, India; IQ, Iraq; IR, Iran; JO, Jordan; KR, Korea; KW, Kuwait; NC, Nanchang; PK, Pakistan; SA, Saudi Arabia; ST, Shantou; SH, Shanghai; WI, Wisconsin. [(b)]The highest percentage of homology are shown in bold.

Viruses	Gene	H9N2									H7N3
		Ck/SA/CP 7/98[(a)]	Ck/AE/R6 6/02	Ck/PK/2/ 99	Ck/PK/U DL-01/06	Ck/PK/U DL-01/08	HK/1073/ 99	Q[(a)]/HK/ G1/97	Dk/HK/Y 280/97	Pa/CH/1/9 7	Ck/PK/NA RC-100/04
Ck/IR/ ZMT-101/98	PB2	**99.6** [(b)]	90.0	88.8	90.1	89.7	89.1	89.1	86.1	89.3	90.0
	PB1	**99.6**	98.6	91.4	92.6	92.4	91.5	91.6	91.7	91.6	92.7
	PA	**99.1**	95.9	90.0	94.2	93.4	89.7	90.0	89.0	90.0	93.9
	NP	**99.6**	93.1	94.2	93.5	93.0	93.9	94.4	91.6	94.5	92.9
Ck/IR/ EBGV-88/10	PB2	89.1	93.6	86.6	**96.9**	96.1	86.8	86.7	86.0	87.5	90.0
	PB1	92.7	92.0	90.2	**98.2**	96.8	90.2	90.2	89.6	90.2	96.0
	PA	92.7	94.0	88.0	95.5	**97.2**	87.9	88.1	87.3	88.0	94.8
	NP	92.1	94.1	95.1	98.1	**98.2**	94.9	95.0	88.9	95.3	94.7

Results

Phylogenetic Analysis: Figure 1 shows the phylogenetic relationship of RNP complex genes of H9N2 subtype of AIVs. Phylogenetic analysis of RNP complex genes revealed that H9N2 viruses from poultry in Iran have undergone reassortment to generate novel genotypes. In the PB2 phylogenetic tree, Ck/IR/ZMT-101/98 isolate with other Iranian and the Middle Eastern isolates fell into domestic duck gene pool (Dk1), while Ck/IR/EBGV-88/10 isolate is related to Pakistani isolates (with more than 96.1% of similarity) and has formed a group from an unknown avian source.

The Nucleotide sequence analysis of PB1 genes showed that Ck/IR/ZMT-101/98 and Ck/IR/EBGV-88/10 shared closest relationship with H9N2 isolates from Ck/SA/CP7/98 and Ck/PK/UDL-01/06, respectively (Table 1). In the PB1 gene tree, the studied isolates were categorized in two different unknown groups. It was of note that Ck/IR/EBGV-88/10 showed evidence of reassortment with other highly pathogenic avian influenza (HPAI). It was 96.0% similar to Ck/PK/NARC-100/04, a H7N3 subtype isolated from Pakistan (Table 1).

The PA genes of Ck/IR/ZMT-101/98 and Ck/IR/EBGV-88/10 strains fell into two separate groups, without any known source for these genes. Besides, the H9N2 Iranian strains shared 97 to 99 % similarity with other isolates such as Ck/PK/UDL-01/08 and Ck/SA/CP7/98 strains (Table 1).

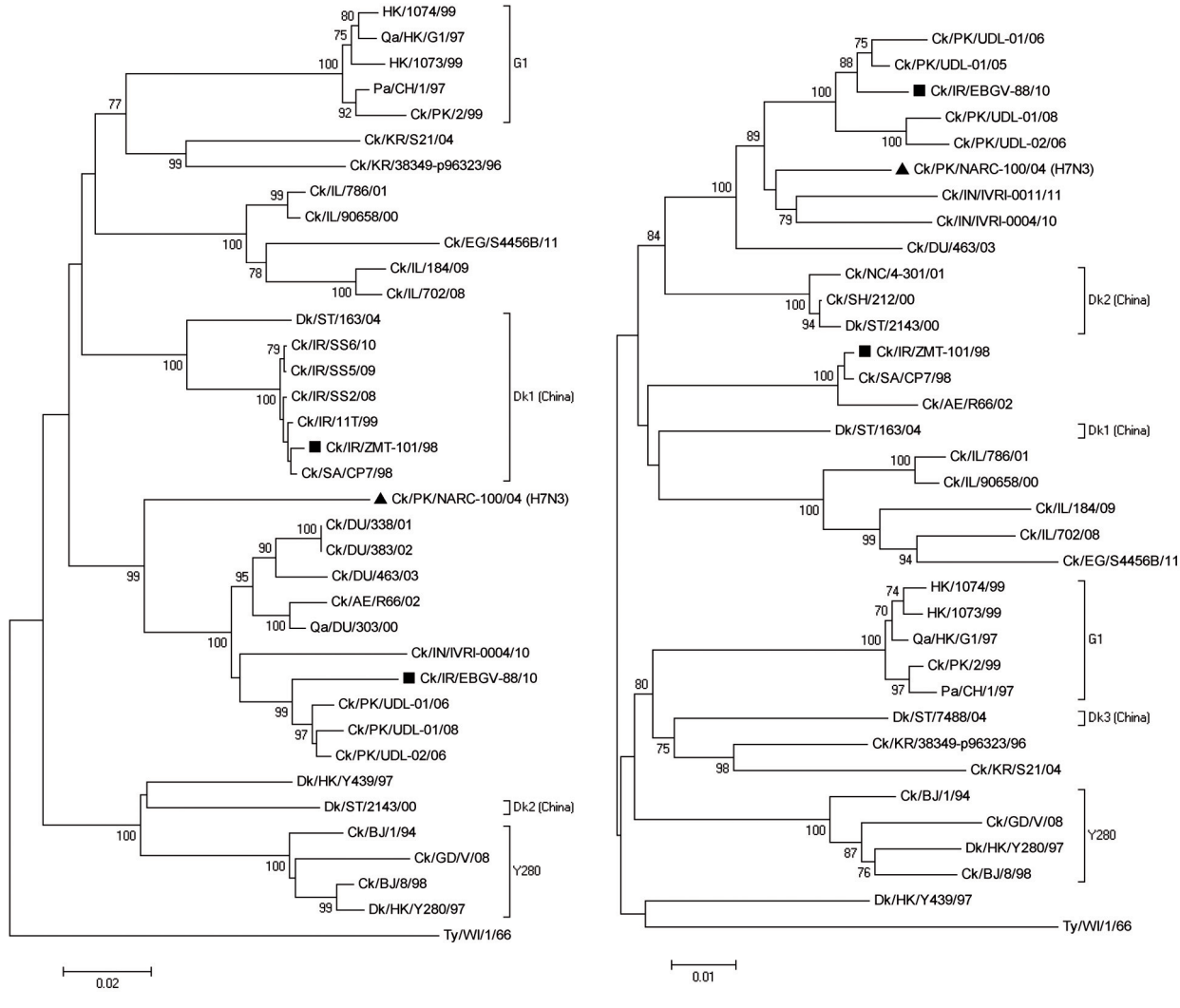
In the NP trees, the new isolate, Ck/IR/EBGV-88/10, was grouped in G1 sub-lineage with isolates from Pakistan and shared a common ancestor with Qa/HK/G1/97 isolate which had contributed internal genes of H5N1 virus from human and avian isolates; however, Ck/IR/ZMT-101/98 fell into Dk sub-lineage with other Middle Eastern isolates. It was most homologous to Saudi Arabian isolate, Ck/SA/CP7/98, another H9N2 virus of Dk sub-lineage.

Analysis and Comparison of Nucleotide and Deduced Amino Acid Sequences of RNP Complex Genes: The deduced amino acid sequences of RNP complex genes were aligned and compared with those of other H9N2 reference viruses. Most of the numerous residues of host associated genetic signatures are located in the RNP complex genes (Table 2). Some positions within the RNP complex genes have been shown to be specific to host species while others can influence virulence in host species. The analysis of these positions revealed that both viruses possessed the typical avian residue at each of these locations except positions 13 in PB1 (L➔P in both strains), 356 in PA protein (L ➔ R in Ck/ IR/ EBGV-88/10) and residues 136 (L ➔ M in Ck/IR/ ZMT-101/98) and 372 (D ➔ E in Ck/IR/EBGV-88/ 10) in the NP protein, which showed the human host signatures. The proline at position 13 of PB1 is common within H9N2 viruses.

PB1-F2 is a small protein of up to 90 amino acids that is encoded by an additional open reading frame (ORF) overlapping the PB1 genes. In the present

Table 2. Molecular host adaptation and virulence markers of RNP complex genes in H9N2 viruses isolated from poultry in Iran. [(a)] Dominant amino acid residues are shown in this table. [(b)] Virulence markers are shown in bold.

Protein	Amino acid position	Amino acid present in Iranian isolates		Predicted amino acid[(a)]		Reference
		Ck/IR/ZMT-101/98	Ck/IR/EBGV-88/10	Avian residue	Mammalian residue	
PB2	44	A	A	A	S	(Naffakh et al., 2008)
	81	T	T	T	M	(Shaw et al., 2002)
	199	A	A	A	S	(Naffakh et al., 2008)
	256[(b)]	D	D	D	G	(Manzoor et al., 2009)
	271	T	T	T	A	(Naffakh et al., 2008)
	333	T	T	T	I	(Naffakh et al., 2008)
	355	R	R	K	Q	(Katz et al., 2000)
	475	L	L	L	M	(Naffakh et al., 2008)
	482	K	K	K	R	(Naffakh et al., 2008)
	567	D	D	D	N	(Taubenberger et al., 2005)
	588	V	A	A	I	(Naffakh et al., 2008)
	613	V	V	V	T	(Naffakh et al., 2008)
	627	E	E	E	K	(Naffakh et al., 2008)
	661	A	A	A	T	(Shaw et al., 2002)
	674	A	A	A	T	(Naffakh et al., 2008)
	701	D	D	D	N	(Naffakh et al., 2008)
	702	K	K	K	R	(Shaw et al., 2002)
	714	S	S	S	R	(Naffakh et al., 2008)
PB1	**13**	P	P	L	P	(Naffakh et al., 2008)
	327	R	R	R	K	(Naffakh et al., 2008)
	336	V	V	V	I	(Naffakh et al., 2008)
	375	N	N	N	S	(Taubenberger et al., 2005)
	538	D	D	D	G	(Naffakh et al., 2008)
	578	K	K	K	Q	(Naffakh et al., 2008)
	614	E	E	E	G	(Naffakh et al., 2008)
	678	S	S	S	N	(Naffakh et al., 2008)
PB1-F2	73	K	K	K	R	(Chen et al., 2006)
	76	V	V	V	A	(Chen et al., 2006)
	79	R	R	R	Q	(Chen et al., 2006)
	82	L	L	L	S	(Chen et al., 2006)
	87	E	E	E	G	(Chen et al., 2006)
PA	28	P	P	P	L	(Naffakh et al., 2008)
	55	D	D	D	N	(Naffakh et al., 2008)
	57	R	R	R	Q	(Naffakh et al., 2008)
	65	P	S	S	L/Y	(Naffakh et al., 2008)
	100	V	I	V	A	(Shaw et al., 2002)
	133	E	E	E	G	(Naffakh et al., 2008)
	225	S	S	S	C	(Naffakh et al., 2008)
	241	C	C	C	Y	(Shaw et al., 2002)
	268	L	L	L	I	(Naffakh et al., 2008)
	312	K	K	K	R	(Shaw et al., 2002)
	356	K	R	K	R	(Naffakh et al., 2008)
	382	E	E	E	D	(Naffakh et al., 2008)
	400	S	S	Q/T/S	L	(Shaw et al., 2002)
	404	A	A	A	S	(Naffakh et al., 2008)
	409	S	S	S	N	(Naffakh et al., 2008)
	552	T	T	T	S	(Naffakh et al., 2008)
	556	Q	Q	Q	R	(Naffakh et al., 2008)
	615	K	K	K	N	(Naffakh et al., 2008)
NP	16	G	G	G	D	(Naffakh et al., 2008)
	31	R	R	R	K	(Shaw et al., 2002)
	33	V	V	V	I	(Naffakh et al., 2008)
	34	G	G	D	N	(Naffakh et al., 2008)
	61	I	I	I	L	(Naffakh et al., 2008)
	100	R	R	R	V	(Naffakh et al., 2008)
	109	I	I	I	V	(Naffakh et al., 2008)
	127	E	E	E	D	(Shaw et al., 2002)
	136	M	I	L	M	(Shaw et al., 2002)
	214	R	R	R	K	(Naffakh et al., 2008)
	283	L	L	L	P	(Naffakh et al., 2008)
	293	R	R	R	K	(Naffakh et al., 2008)
	305	R	R	R	K	(Naffakh et al., 2008)
	313	F	F	F	Y	(Naffakh et al., 2008)
	319	N	N	N	K	(Naffakh et al., 2008)
	357	Q	Q	Q	K	(Naffakh et al., 2008)
	372	E	D	E	D	(Naffakh et al., 2008)
	375	D	D	D	G/E	(Shaw et al., 2002)
	422	R	R	R	K	(Naffakh et al., 2008)
	442	T	T	T	A	(Naffakh et al., 2008)
	455	D	D	D	E	(Naffakh et al., 2008)
	480	D	D	D	N	(Naffakh et al., 2008)



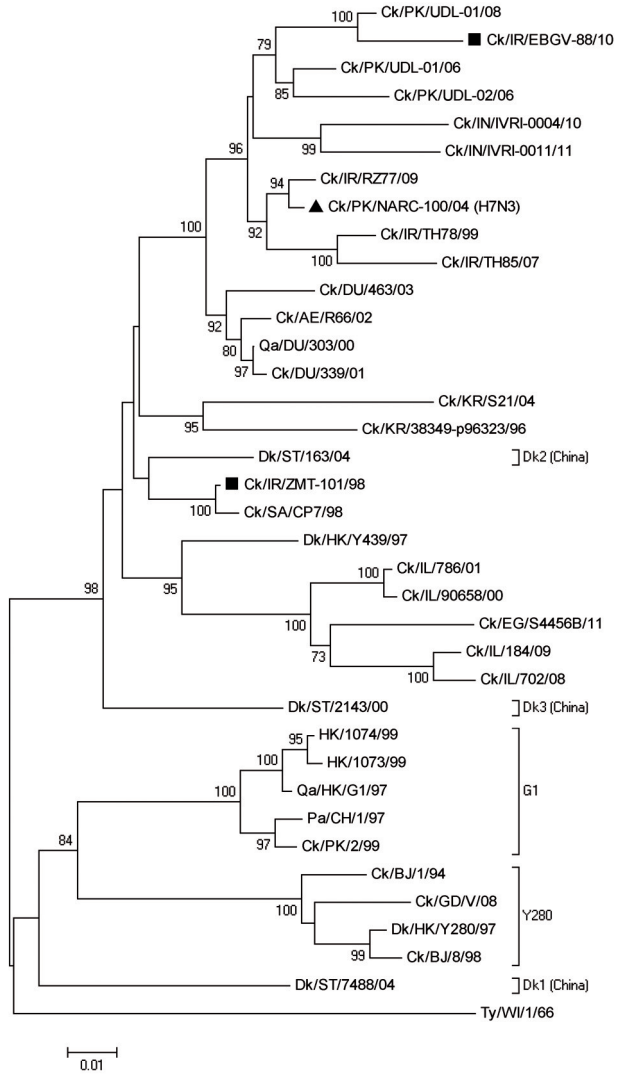
(a) PB2

(b) PB1

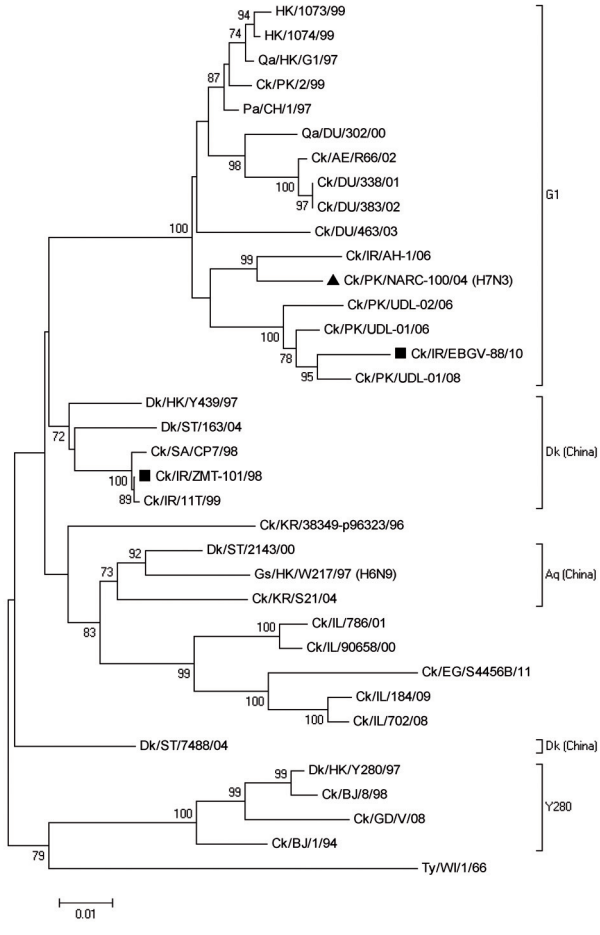
Figure 1. Phylogenetic analysis of the (a) PB2, (b) PB1, (c) PA and (d) NP genes of Iranian influenza A viruses. The phylogenetic trees were generated by the distance-based neighbor-joining method with MEGA 5.0 program. The nucleotide sequences used for the phylogenetic analysis are as follows: PB2 (53-2289), PB1 (46-2230), PA (25-2166) and NP (46-1491). Numbers near the nodes show the percentage of the bootstrap values of 1000 replicates. Viruses characterized in this study are marked with square and H7N3 are indicated by triangle. Abbreviations can be found in table 1.

study, the deduced amino acid sequences of both analyzed H9N2 viruses have 90 amino acids similar to Qa/HK/G1/97. None of the tested strains showed serine at position 66 of the PB1-F2 protein; however, viruses with an N66S mutation in PB1-F2 caused increased virulence in mice.

Both of the studied H9N2 viruses displayed glutamic acid at position 627 of the PB2 protein that is similar to avian viruses. A PB2 D701N also could serve as a marker of increased influenza virus virulence in mammals. The current Iranian strains carried D701 in PB2 protein.



(c) PA



(d) NP

Figure 1. continued.

Discussion

H9N2 viruses have circulated widely in Iran and are associated with disease in commercial poultry. Recent studies have shown that H9N2 viruses from Iran have been able to bind to α2,6-linked sialic acid receptors on human respiratory cells, suggesting the possibility that H9N2 has the potential to cause a human pandemic (Homayounimehr et al., 2010). There is also evidence of seropositivity against H9N2 virus among poultry workers in Iran (Alizadeh et al., 2009). Furthermore, it has been reported that transmission of H9N2 from avian to human can occur, which increased the concern about their pandemic potential to humans (Lin et al., 2000). The molecular mechanisms by which H9N2 influenza viruses transmit from avian to humans are not fully

understood. Numerous substitutions in RNP complex have been recognized as an important contributor to alter the host range and virulence of AIVs. Therefore, understanding the genetic characteristic of RNP complex genes can provide insight into genotypic signatures of pathogenicity and the monitoring of the pandemic potential of AIVs. In the present study, the genetic basis underlying the virulence and host range of RNP complex genes of two H9N2 influenza viruses in chickens have been reported.

Previous studies have identified two distinct lineages in H9N2 influenza viruses of North America and Eurasian lineages. Subsequently, the Eurasian lineage has been classified into two major sub-lineages including the G1 sub-lineage, represented by Qa/HK/G1/97; and the Y280 sub-lineage, represented by Dk/HK/Y280/97 (Guan et al., 2000; Xu et al., 2007). In the long-term influenza virus surveillance in China, ribonucleoprotein complex genes had more diversified sources than surface genes. In addition to G1 and Y280 sub-lineages, there have been different sub-lineages, including duck (Dk1, Dk2 and Dk3), aquatic birds, Gs/Gd-like and H5N1/01-like sub-lineages (Xu et al., 2007). In the present study, the phylogenetic analysis of RNP complex genes revealed that these genes have undergone genetic reassortment. The polymerase complex (PB2, PB1 and PA) of Ck/IR/EBGV-88-10 fell into unknown avian groups, while the NP gene of this strain was similar to those of other Middle Eastern strains, which drived from the G1 sub-lineage. The PB1 and PA genes of Ck/IR/ZMT-101/98 clustered into an unknown source and PB2 and NP genes of this isolate formed a group with other Iranian and Middle Eastern isolates which likely derived from ducks in China (Dk1 and Dk sub-lineages, respectively) (Xu et al., 2007).

A recent large evolutionary study (Fusaro et al., 2011) demonstrated that H9N2 influenza viruses consisted of four distinct and co-circulating groups (A to D) in the Middle Eastern and Central Asian countries from 1998 to 2010. Group D and B included viruses from Iran isolated in the years between 1998 and 2007, and 2008-2009, respectively. The HA, NA and M genes of the four genetic groups, as well as the NP gene of group B, fell into the G1 sub-lineage, while the remaining genes were clustered with other distinct sub-lineages, and did not show any relationship with the previously identified G1 and Y280 sub-lineages. Our findings were in accordance with this research since they revealed that RNP complex genes showed no clear geographic sub-lineages.

In the current study, PB1, PA and NP genes of Ck/IR/EBGV-88-10 were closely related (94.7-96%) to a HPAI virus, Ck/PK/NARC-100/04 (H7N3). It was of note that this virus from Pakistan shared an out-group relationship with Ck/IR/EBGV-88/10 and other Middle Eastern strains, suggesting that these viruses were derived from the same gene pool. Previous studies (Iqbal et al., 2009) showed that some H9N2 viruses isolated in Pakistan have acquired the NS gene from HPAI subtypes H5 and H7. On the basis of the similarity indices and phylogenetic analysis of RNP complex genes, it can be argued that the H9N2 viruses from Iran have undergone genetic reassortment with other AIVs.

Although HA plays a critical role in virulence or pathogenicity of AIVs in birds, its pathogenicity is considered as a multigenic trait; it is determined by a complete set of genes within a particular influenza virus strain in a specific host (Perdue, 2008). Among the virulence and host range determinants, numerous substitutions have been identified on the RNP complex genes.

Regarding polymerase complex, the PB2 protein has been recognized as an important contributor to viral pathogenicity. The genetic analysis of PB2 revealed that both isolates carried glutamic acid at position 627. Within PB2, amino acid at position 627 is always glutamic acid in avian isolates, whereas nearly all human isolates carry lysine at this position. A PB2 E627K mutation in H5N1 subtype strain caused a lethal, systemic infection in mice, but became non pathogenic for mammals if the residue remained a glutamic acid (Steel et al., 2009). Moreover, D701N mutation is also associated with increased virulence of AIV within the PB2 subunit. Both of the studied isolates detected aspartic acid at position 701 of PB2 found in AIVs isolated from birds (Gao et al., 2009).

In PB1 and PA genes, the other two proteins of the polymerase complex have also been shown to have roles in determination of host range. The studied Iranian isolates showed the typical avian residue at each of these positions except position 13 in PB1

which is common among H9N2 viruses.

Full length PB1-F2 proteins (≥79 amino acids) are expressed by nearly all avian influenza viruses and tend to become truncated following introduction into mammalian hosts (McAuley et al., 2010). The PB1-F2 protein of Iranian isolates was expressed in full length. The expression of full length PB1-F2 has been associated with increased virulence and pathogenicity of influenza A virus. In addition, viruses with an N66S mutation caused increased disease severity, lung titers and cytokine production in mice (Conenello et al., 2007), but viruses with S66 were rare and thus not present in the H9N2 viruses of this study.

Nucleoprotein (NP) plays an essential role in the structural organization of the RNP complex and is required for the transcription and replication of the viral genome. It interacts with a wide variety of viral and cellular macromolecules, including PB2, PB1, M1 and cellular polypeptides for viral transcription and nuclear transportation controls (Portela and Digard, 2002). The phylogenetic and genetic analysis of the swine, avian and human influenza viruses confirmed the highly conserved nature of the NP gene within host specific lineages, hence suggesting its role in determining species specificity (Thippamom et al., 2010). Among the 22 residues associated with host range, 20 residues were identical in the studied isolates and showed the avian host signature. The exceptions were L136M in Ck/IR/ZMT-101/98 and D372E in Ck/IR/EBGV-88/10 strains. Whether these changes result in increased replication efficiency in mammalian cells is unclear and remains to be determined.

The reassortment of viral gene segments is one of the mechanisms of influenza virus evolution. In a recent panorama phylogenetic analysis, it has been reported that internal genes of H9N2 viruses are closely related to H3, H4, H5, H7, H10 and H14 subtypes of influenza viruses (Dong et al., 2011). In addition, the first human H9N2 viruses are similar to strain Qa/HK/G1/97 that shares internal genes with H5N1 viruses responsible for 1997 Hong Kong H5N1 virus (Lin et al., 2000). These findings suggest that H9N2 viruses have undergone extensive reassortments to generate multiple reassortants and genotypes (Dong et al., 2011; Fusaro et al., 2011).

In this study, the complete sequence analysis of RNP complex genes of H9N2 subtype of AIVs isolated from chickens in Iran has been reported for the first time. These results demonstrate that RNP complex genes are derived from different sub-lineages, suggesting genetic reassortment of the Iranian viruses with other AIVs. In addition to the widespread occurrence of the H9N2 viruses, outbreaks of highly pathogenic H5N1 subtype in wild birds and backyard chickens in 2006 and 2008, respectively, in North of Iran (Fereidouni et al., 2010), highlight the need for continuous monitoring of AIVs which are considered to have potential for the evolution of this virus.

Acknowledgments

This study was supported by a grant (No. 7508001/6/6) from the Research Council of Tehran University.

References

1. Alexander, D.J. (2007) Summary of avian influenza activity in Europe, Asia, Africa, and Australasia, 2002-2006. Avian Dis. 51: 161-166.
2. Alizadeh, E., Hosseini, S.E., Kheiri, M.T., Mazaheri, V., Bashar, R., Tabatabaeian, M. (2009) Avian influenza (H9N2) among poultry workers in Iran. Iran J Microbiol. 1: 3-6.
3. Chen, G.W., Chang, S.C., Mok, C.K., Lo, Y.L., Kung, Y.N., Huang, J.H., Shih, Y.H., Wang, J.Y., Chiang, C., Chen, C.J., Shih, S.R. (2006) Genomic signatures of human versus avian influenza A viruses. Emerg Infect Dis. 12: 1353-1360.
4. Conenello, G.M., Zamarin, D., Perrone, L.A., Tumpey, T., Palese, P. (2007) A single mutation in the PB1-F2 of H5N1 (HK/97) and 1918 influenza A viruses contributes to increased virulence. PLoS Pathog. 3: 1414-1421.
5. Dong, G., Xu, C., Wang, C., Wu, B., Luo, J., Zhang, H., Nolte, D.L., Deliberto, T.J., Duan, M., Ji, G., He, H. (2011) Reassortant H9N2 influenza viruses containing H5N1-like PB1 genes isolated from black-billed magpies in Southern China. PloS one. 6: e25808.
6. Fereidouni, S.R., Werner, O., Starick, E., Beer, M., Harder, T.C., Aghakhan, M., Modirrousta, H., Amini, H., Moghaddam, M.K., Bozorghmehrifard, M.H.,

Akhavizadegan, M.A., Gaidet, N., Newman, S.H., Hammoumi, S., Cattoli, G., Globig, A., Hoffmann, B., Sehati, M.E., Masoodi, S., Dodman, T., Hagemeijer, W., Mousakhani, S., Mettenleiter, T.C. (2010) Avian influenza virus monitoring in wintering waterbirds in Iran, 2003-2007. Virol J. 7: 43.

7. Fusaro, A., Monne, I., Salviato, A., Valastro, V., Schivo, A., Amarin, N.M., Gonzalez, C., Ismail, M.M., Al-Ankari, A.R., Al-Blowi, M.H., Khan, O.A., Maken Ali, A.S., Hedayati, A., Garcia Garcia, J., Ziay, G.M., Shoushtari, A., Al Qahtani, K.N., Capua, I., Holmes, E.C., Cattoli, G. (2011) Phylogeography and evolutionary history of reassortant H9N2 viruses with potential human health implications. J Virol. 85: 8413-8421.
8. Gao, Y., Zhang, Y., Shinya, K., Deng, G., Jiang, Y., Li, Z., Guan, Y., Tian, G., Li, Y., Shi, J., Liu, L., Zeng, X., Bu, Z., Xia, X., Kawaoka, Y., Chen, H. (2009) Identification of amino acids in HA and PB2 critical for the transmission of H5N1 avian influenza viruses in a mammalian host. PLoS Pathog. 5: e1000709.
9. Guan, Y., Shortridge, K.F., Krauss, S., Chin, P.S., Dyrting, K.C., Ellis, T.M., Webster, R.G., Peiris, M. (2000) H9N2 influenza viruses possessing H5N1-like internal genomes continue to circulate in poultry in southeastern China. J Virol. 74: 9372-9380.
10. Hoffmann, E., Stech, J., Guan, Y., Webster, R.G., Perez, D.R. (2001) Universal primer set for the full-length amplification of all influenza A viruses. Arch Virol. 146: 2275-2289.
11. Homayounimehr, A.R., Dadras, H., Shoushtari, A., Pourbakhsh, S.A. (2010) Sequence and phylogenetic analysis of the haemagglutinin genes of H9N2 avian influenza viruses isolated from commercial chickens in Iran. Trop Anim Health Prod. 42: 1291-1297.
12. Iqbal, M., Yaqub, T., Reddy, K., McCauley, J.W. (2009) Novel genotypes of H9N2 influenza A viruses isolated from poultry in Pakistan containing NS genes similar to highly pathogenic H7N3 and H5N1 viruses. PloS one. 4: e5788.
13. Katz, J.M., Lu, X., Tumpey, T.M., Smith, C.B., Shaw, M.W., Subbarao, K. (2000) Molecular correlates of influenza A H5N1 virus pathogenesis in mice. J Virol. 74: 10807-10810.
14. Lee, M.S., Chang, P.C., Shien, J.H., Cheng, M.C., Shieh, H.K. (2001) Identification and subtyping of avian influenza viruses by reverse transcription-PCR. J Virol Methods. 97: 13-22.
15. Lin, Y.P., Shaw, M., Gregory, V., Cameron, K., Lim, W., Klimov, A., Subbarao, K., Guan, Y., Krauss, S., Shortridge, K., Webster, R., Cox, N., Hay, A. (2000) Avian-to-human transmission of H9N2 subtype influenza A viruses: relationship between H9N2 and H5N1 human isolates. Proc Natl Acad Sci. 97: 9654-9658.
16. Manzoor, R., Sakoda, Y., Nomura, N., Tsuda, Y., Ozaki, H., Okamatsu, M., Kida, H. (2009) PB2 protein of a highly pathogenic avian influenza virus strain A/chicken/Yamaguchi/7/2004 (H5N1) determines its replication potential in pigs. J Virol. 83: 1572-1578.
17. Matrosovich, M.N., Krauss, S., Webster, R.G. (2001) H9N2 influenza A viruses from poultry in Asia have human virus-like receptor specificity. Virology. 281: 156-162.
18. McAuley, J.L., Chipuk, J.E., Boyd, K.L., Van De Velde, N., Green, D.R., McCullers, J.A. (2010) PB1-F2 proteins from H5N1 and 20 century pandemic influenza viruses cause immunopathology. PLoS Pathog. 6: e1001014.
19. Naeem, K., Ullah, A., Manvell, R.J., Alexander, D.J. (1999) Avian influenza A subtype H9N2 in poultry in Pakistan. Vet Rec. 145: 560.
20. Naffakh, N., Tomoiu, A., Rameix-Welti, M.A., van der Werf, S. (2008) Host restriction of avian influenza viruses at the level of the ribonucleoproteins. Ann Rev Microbiol. 62: 403-424.
21. Perdue, M.L. (2008) Molecular determinants of pathogenicity for avian influenza viruses In: Avian influenza. Swayne, D.E. (ed.). Blackwell Publishing Ltd London, UK. p. 23-42.
22. Portela, A., Digard, P. (2002) The influenza virus nucleoprotein: a multifunctional RNA-binding protein pivotal to virus replication. J Gen Virol. 83: 723-734.
23. Qiu, B.F., Liu, W.J., Peng, D.X., Hu, S.L., Tang, Y.H., Liu, X.F. (2009) A reverse transcription-PCR for subtyping of the neuraminidase of avian influenza viruses. J Virol Methods. 155: 193-198.
24. Shaw, M., Cooper, L., Xu, X., Thompson, W., Krauss, S., Guan, Y., Zhou, N., Klimov, A., Cox, N., Webster, R., Lim, W., Shortridge, K., Subbarao, K. (2002) Molecular changes associated with the transmission of avian influenza a H5N1 and H9N2 viruses to humans. J Med Virol. 66: 107-114.
25. Steel, J., Lowen, A.C., Mubareka, S., Palese, P.

(2009) Transmission of influenza virus in a mammalian host is increased by PB2 amino acids 627K or 627E/701N. PLoS Pathog. 5: e1000252.

26. Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S. (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol. 28: 2731-2739.
27. Taubenberger, J.K., Reid, A.H., Lourens, R.M., Wang, R., Jin, G., Fanning, T.G. (2005) Characterization of the 1918 influenza virus polymerase genes. Nature. 437: 889-893.
28. Thippamom, N., Sreta, D., Kitikoon, P., Thanawongnuwech, R., Poovorawan, Y., Theamboonlers, A., Suwannakarn, K., Parchariyanon, S., Damrongwatanapokin, S., Amonsin, A. (2010) Genetic variations of nucleoprotein gene of influenza A viruses isolated from swine in Thailand. Virol J. 7: 185.
29. Vasfi Marandi, M., Bozorgmehri Fard, M.H. (2002) Isolation of H9N2 Subtype of Avian Influenza Viruses during an Outbreak in Chickens in Iran. Iran Biomed J. 6: 13-17.
30. Vasfi Marandi, M., Bozorgmehri Fard, M.H., Hashemzadeh, M. (2002) Efficacy of inactivated H9N2 avian influenza vaccine against non-highly pathogenic A/ Chicken/ Iran/ ZMT-173/ 1999 infection. Arch Razi Inst. 53: 23-31.
31. Xu, K.M., Li, K.S., Smith, G.J., Li, J.W., Tai, H., Zhang, J.X., Webster, R.G., Peiris, J.S., Chen, H., Guan, Y. (2007a) Evolution and molecular epidemiology of H9N2 influenza A viruses from quail in southern China, 2000 to 2005. J Virol. 81: 2635-2645.
32. Xu, K.M., Smith, G.J., Bahl, J., Duan, L., Tai, H., Vijaykrishna, D., Wang, J., Zhang, J.X., Li, K.S., Fan, X.H., Webster, R.G., Chen, H., Peiris, J.S., Guan, Y. (2007b) The genesis and evolution of H9N2 influenza viruses in poultry from southern China, 2000 to 2005. J Virol. 81: 10389-10401.

مجله طب دامی ایران، ۱۳۹۲، دوره ۷، شماره ۳، ۱۶۸-۱۵۹

آنالیز ژنتیک و فیلوژنتیک ژن های ریبونوکلئوپروتئین ویروس های H9N2 آنفلوانزای پرندگان جدا شده از طیور تجاری در ایران

محسن بشاشتی[۱] مهدی وصفی مرندی[۱*] محمد حسن بزرگمهری فرد[۱] فرهید همت زاده[۲] فرشته صبوری[۱]

۱) گروه بیماریهای طیور، دانشکده دامپزشکی دانشگاه تهران، تهران، ایران

۲) دانشکده دامپزشکی و علوم دامی، دانشگاه آدلاید، آدلاید، استرالیا

(دریافت مقاله: ۱۹ بهمن ماه ۱۳۹۱ ، پذیرش نهایی: ۱۵ اردیبهشت ماه ۱۳۹۲)

چکیده

زمینه مطالعه: تحت تیپ H9N2 ویروس های آنفلوانزای پرندگان (AIVs) از گونه های مختلف پرندگان در اکثر کشورهای اروپایی، آسیایی، آفریقایی و آمریکایی جدا شده است. پس از اولین شیوع ویروس H9N2 در سال ۱۹۹۸ در ایران، این ویروس به طور گسترده در تمام کشور گسترش یافته است و موجب ضررهای بزرگ اقتصادی در گله های طیور گردیده است. اسیدهای آمینه متعددی در کمپلکس ریبونوکلئوپروتئین (RNP) که شامل پروتئین های NP و پلیمرازی (PB2، PB1 و PA) است، مرتبط با طیف میزبانی و حدت می باشند. **هدف:** هدف ما در این مطالعه، تعیین هویت مولکولی کمپلکس RNP در جدایه های ایرانی تحت تیپ H9N2 می باشد. **روش کار:** تمام طول توالی نوکلئوتیدی کمپلکس RNP دو سویه به عنوان Ck/IR/ZMT-101/98 و Ck/IR/EBGV-88/10 تکثیر و توالی یابی گردید. **نتایج:** آنالیز فیلوژنتیک نشان داد که هر دو سویه در زیر دسته های مختلف قرار می گیرند. براساس شباهت های ژنتیکی، ژن های PB1، PA و NP سویه Ck/IR/EBGV-88/10 ارتباط نزدیکی با یک تحت H7N3 جدا شده از پاکستان داشت. اکثر جایگاه های دامنه میزبانی دارای اسیدآمینه مرتبط با تعیین کننده های پرندگان در پروتئین RNP بود. **نتیجه گیری نهایی:** براساس این نتایج، ژن های RNP ایران دچار بازآرایی ژنتیکی شده اند. پایش مداوم AIV در صنایع طیور به منظور بدست آوردن اطلاعات بیشتر در رابطه با تنوع ژنتیکی ویروس های H9N2 و ظهور احتمالی ویروس های حاد و یا پندمی کمک کننده می باشد.

واژه های کلیدی: ویروس آنفلوانزای طیور، H9N2، ریبونوکلئوپروتئین، طیور

*)نویسنده مسؤول: تلفن: ۶۶۹۲۳۵۱۰ (۲۱) ۹۸+ نمابر: ۶۶۹۳۳۲۲۲ (۲۱) ۹۸+ *Email: mvmarand@ut.ac.ir*