

Phylogenetic analysis of PB2 gene of H9N2 subtype of avian influenza viruses isolated from commercial chickens in Tehran province of Iran during 1998-2001

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Summary

The PB2 protein of influenza A viruses has been shown as host range determinant. In this study PB2 gene of five H9N2 isolates from Tehran province during the period of 1998-2001 were partially amplified and sequenced to do phylogenetic study. Iranian isolates could be classified in Eurasian lineage according to their PB2 gene sequences. The PB2 genes of these isolates are different from those of Middle-East countries such as the UAE, Pakistan and Saudi Arabia, as well as India, and it is probable that their ancestors have undergone reassortment with other subtypes. The Nucleotide sequences of their PB2 are highly similar to each other with 97.5-99.6% homology percentage showing the least mutations in their genes during the period of 1998 to 2001. The PB2 of the Iranian isolates contain Glutamic acid-(E) in position 627, Aspartic acid-(D) in 701 and Serine-(S) in 714 as other avian isolates.

Key words: Avian influenza, H9N2, PB2, Phylogenetic analysis

Introduction

H9N2 subtype of avian influenza (AI) virus was first isolated in Iran in 1998 and has been grouped as non-highly pathogenic (Vasfi Marandi and Bozorgmehri Fard, 2002; Kianizadeh *et al.*, 2006; Toroghi and Momayez, 2006). H9N2 viruses have been isolated from several areas of Asia such as Honk Kong, Pakistan, China, Iran, Saudi Arabia and the United Arab Emirates. Human cases infected by H9N2 documented in Hong Kong and China showed that these viruses could be transmitted directly from birds to human (Peiris *et al.*, 1999; Guan *et al.*, 2000). The characteristics of two hemagglutinin (HA) and polymerase basic 2 (PB2) proteins of AI viruses have been demonstrated as the main determinants of host range (Subbarao *et al.*, 1993; Hatta *et al.*, 2000; Gabriel *et al.*, 2005, 2007; Labadie *et al.*, 2007).

Three polymerases (PB1, PB2 and PA), the nucleoprotein (NP), and the viral RNAs

compose the ribonucleoprotein (RNP) complex of influenza A viruses. The polymerases of this complex are responsible for the transcription and replication of viral RNAs. RNP complexes must be transported to and operate in the host cell nucleus (Gorman *et al.*, 1990). The PB1 subunit is the core of the polymerase complex and as a central part of it is responsible for RNA synthesis (Perales *et al.*, 1996). Induction of a proteolysis in cells expressing the PA protein by transfection has been described (Perales *et al.*, 1996). The PB2 subunit has been shown to interact with cap-1 structures and presumably recruits capped RNA in the nucleus of the infected cell. It has been suggested that the PB2 subunit acts as an endonuclease in the generation of transcription primers, but the enzymatic activity requires the association of all three polymerase subunits (Perales *et al.*, 1996).

There is growing evidence that enhanced polymerase activities facilitate species transmission and increase virulence in

mammals (Subbarao *et al.*, 1993). The substitution of lysine in position 627 in PB2 protein is known in human infecting virus strains (Subbarao *et al.*, 1993) including H1N1 subtype of the year 1918 (Taubenberger *et al.*, 2005) and H5N1 subtype of 1997 as well as the H7N7 HPAI isolate (Hatta *et al.*, 2000).

In several studies it has been shown that the HA antigen of most of the Iranian H9N2 isolates have gotten the characteristics to bind to human-type receptors by substitution of the amino acid Leucine-(L) instead of Glutamine-(Q) at position 226 (Homayounimehr *et al.*, 2010; Moosakhani *et al.*, 2010). In this study the PB2 gene of H9N2 isolates of Tehran province was partially amplified and sequenced. The existing evolutions in PB2 and probable changes in amino acid in position 627 and other important reported positions were investigated.

Materials and Methods

Viruses

Four isolates of H9N2 provided by the Veterinary Faculty of the University of Tehran isolated from broiler chickens in Tehran province from 1998 to 2001 were used. The H9N2 isolate of A/Chicken/Iran/ZMT-101/1998(H9N2) isolated from layers in Tehran province in the first outbreaks was sequenced and used in this study (Vasfi Marandi and Bozorgmehri Fard, 2002). The subtype of these isolates was confirmed using a standard hemagglutination inhibition (HI) test as described previously (Alexander and Spackman, 1981).

RNA extraction

Viral RNA was extracted from infected allantoic fluid using RNX-Plus™ (Cinnagen, Iran).

RT and PCR reaction

For reverse transcription (RT), the oligonucleotide influenza universal primer uni 12: 5'-AGC AAA AGC AGG-3' with "Revert aid" first standard cDNA synthesis kit (Fermentas, Canada) was used. A part of 940 bps of the PB2 gene of isolates (about

NT 1400 to 2341) was amplified by RT-PCR (Fig. 1) using one pair of specific primers as described previously (Hoffman *et al.*, 2001). The sequences of the primers used in this study are available on request. The reaction mixture (50 µl) contained 5 µl of cDNA, 15 pmoles of forward and reverse primers (2 µl) and Cinnagen master mix (Cinnagen, Iran). The amplification protocol was: one step denaturation at 94°C for 5 min, 35 cycles of "94°C for 45 Sec, 52°C for 45 Sec and 72°C for 60 Sec" and final extension at 72°C for 10 min. The PCR products were assessed by gel electrophoresis (Fig. 1).

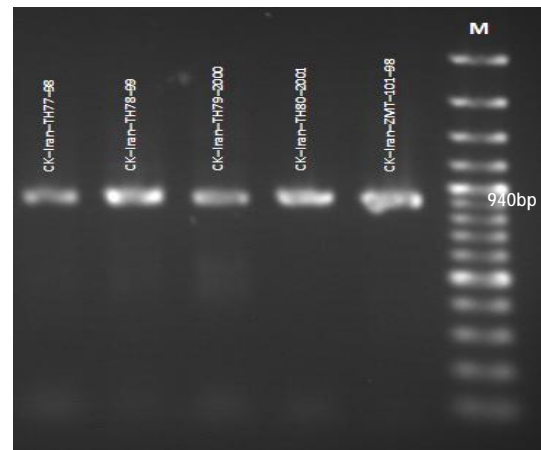


Fig. 1: Gel view for amplified fragment 940 bp of PB2 genes of five H9N2 isolates from Tehran province. M lane shows molecular size marker (100 bp Plus DNA Ladder-GeneRuler™, Fermentas). The 940 bp bands in other five lanes are related to the five H9N2 isolates of Tehran province of Iran as they have appeared in phylogenetic tree in Fig. 3

Sequence analysis and phylogenetic study

The PCR products were sent for purification and direct sequencing from both directions (MWG-Biotech AG, Germany).

The respective nucleotide and deduced amino acid sequences from GenBank, Clustal W method and Megalign™ Software of the DNASTAR package (DNASTAR Inc., version 7, Madison, WI, USA) was used for comparison and determining sequence similarity, the phylogenetic relationships and differences in amino acid sequences of different H9N2 subtype viruses used in this study.

Accession numbers

Five nucleotide sequences obtained in this study are deposited in the GenBank database under accession numbers HM014334 to HM014338.

Results

The positions of amino acid residues were numbered according to the PB2 sequence of A/Turkey/Minnesota/833/1998 (H4N2), (TY/Minnesota/833/98(H4N2) in this study), taken from viruses used by Subbarao *et al.* (1993).

The PB2 of all Iranian H9N2 isolates used in this study and all H9N2 isolates chosen from GeneBank containing H9N2 isolates from Middle-East countries and H9N2 prototypes from different lineages as well as the H4N2 turkey isolate contained

Glutamic acid-(E) in position 627, Aspartic acid-(D) in 701 and Serine-(S) in 714 (Fig. 2). Some amino acid residue substitutions were seen in positions 588, 629, 659 and 667 which are thought to be due to point mutations present in the nucleotide sequences of studied Iranian isolates in comparison with the prototype H9N2, TY/Wisconsin/66 (Fig. 2). However, there is a high amino acid sequence homology of 98.3 to 99.3% among Iranian isolates and this prototype H9N2 virus (Table 1).

The amino acid identity among Iranian isolates was from 98.6 to 100% according to the nucleotide sequence homology extent of 97.5 to 99.6% between them (Tables 1 and 2). Comparison of the identity percentage of nucleotide and amino acid sequences among Iranian isolates with some other isolates from around the world is in Tables 2 and 3.

Table 1: Amino acid homology and divergence percentage between the five Iranian isolates and the prototype H9N2 virus TY/Wisconsin/66

		Percent identity								
		1	2	3	4	5	6	7		
Divergence	1		99.7	99.3	99.3	99.0	100.0	98.7	1	CK-Iran-11T-99
	2	0.3		99.3	99.3	98.6	100.0	98.6	2	CK-Iran-TH77-98
	3	0.7	0.7		100.0	99.7	98.9	98.3	3	CK-Iran-TH78-99
	4	0.7	0.7	0.0		99.7	99.3	98.3	4	CK-Iran-TH79-2000
	5	1.0	1.4	0.4	0.3		98.9	98.3	5	CK-Iran-TH80-2001
	6	0.0	0.0	0.7	0.7	1.1		99.3	6	CK-Iran-ZMT-101-98
	7	1.3	1.0	1.4	1.4	1.4	0.7		7	TY-Wisconsin-66
		1	2	3	4	5	6	7		

Alignment and pair distances computed by Clustal W method. CK: Chicken, and TY: Turkey

Table 2: Homology (%) between the nucleotide and amino acid sequences of the PB2 of five H9N2 isolates of Iran with other countries as they have appeared in phylogenetic tree in Fig. 3

Sequence	IR		PK		UAE		SA		IN		CH		
	NT	AA	NT	AA	NT	AA	NT	AA	NT	AA	NT	AA	
IR	MAX	99.6 ^{a1}	100 ^{b1}	89.3	96.4	90.4	98.9	90.1	97.7	89.8	98.7	87.6	98.6
	MIN	97.5 ^{a2}	98.6 ^{b2}	85.2	94.8	85.6	96.5	81.4	89.5	88.7	97.4	86.9	97.6

^{a1}: CK/Iran/TH77/98 and CK/Iran/11T/99 with CK/Iran/ZMT-101/98. ^{a2}: CK/Iran/TH77/98 with CK/Iran/TH80/2001. ^{b1, b2}: See Table 1. AA: Amino acid, CH: China, IN: India, IR: Iran, MAX: Maximum, MIN: Minimum, PK: Pakistan, SA: Saudi Arabia, UAE: United Arab Emirates, and NT: Nucleotide

Table 3: Homology (%) between the nucleotide and amino acid sequences of the PB2 of five H9N2 isolates of Iran with prototype isolates from different lineages as they have appeared in phylogenetic tree in Fig. 3

Sequence	WIS		G1		Y280		1073		1074		KO		
	NT	AA	NT	AA	NT	AA	NT	AA	NT	AA	NT	AA	
IR	MAX	84.3	99.3	89.1	97.2	86.8	96.9	89.4	97.3	89.2	97.3	90.2	98.6
	MIN	81.6	98.3	83.9	95.2	82.3	94.8	88.2	96.9	87.5	96.9	85.3	97.6

G1: QA/HongKong/G1/97, 1073: HongKong/1073/99, 1074: HongKong/1074/99, KO: CK/Korea/38346-p96323/96, WIS: TY/Wisconsin/66, and Y280: DK/HongKong/Y280/97

Phylogenetic analysis

The phylogenetic tree of the PB2 genes was constructed with sequence data of 800 nucleotides (NT 1450 to 2250) of the five isolates from Iran, 27 PB2 gene sequences containing 26 H9N2 subtype and one H4N2 subtype. In phylogenetic tree four distinct groups can be distinguished (Fig. 3). Group 1 contains “G1 like” H9N2 viruses. Group 3 consists of “Y280 like” viruses.

In group 2 the isolates from the United Arab Emirates (UAE) clustered with the isolate CK/Gujrat/India/369/2004 in a different clade from “G1 like” and “Y280 like” viruses. All Iranian isolates clustered beside each other in one branch in group one close to the G1 like viruses. The isolate 532/99 of Saudi Arabia is in a separate branch in the group 1 closer than Iranian isolates to “G1 like” viruses and its nucleotide sequence identity of 88.4% with QA/HongKong/G1/97 and 89% with CK/HongKong/G9/97 shows almost the same homology as the Iranian isolates have with them (Table 3). The viruses in groups 1, 2 and 3 seem to belong to Eurasian lineage and group 4 containing North American isolates indicates North American

lineage.

Discussion

The H9N2 subtype of avian influenza viruses has become endemic in the poultry population of Asian, Middle East and Eastern European countries (Capua and Alexander, 2004). After the isolation of H9N2 virus from human cases with influenza like symptoms in China and Hong Kong in 1999, it has been considered as an avian virus that, besides the H5N1 subtype, can cross the host barriers to mammals (Perdue and Swayne, 2005). Studies done in Iran indicate the presence of antibodies against H9 in human population. These studies show the ability of Iranian H9N2 subtype to infect human (Rahimian *et al.*, 2009; Moosakhani *et al.*, 2010). Polymerase complex activity, the amino acid sequence of PB2 and the presence of some particular amino acids in some certain positions such as 627, 701 and 714 has been shown as more important host range determinants (Subbarao *et al.*, 1993; Li *et al.*, 2005b; Labadie *et al.*, 2007). In this study it is

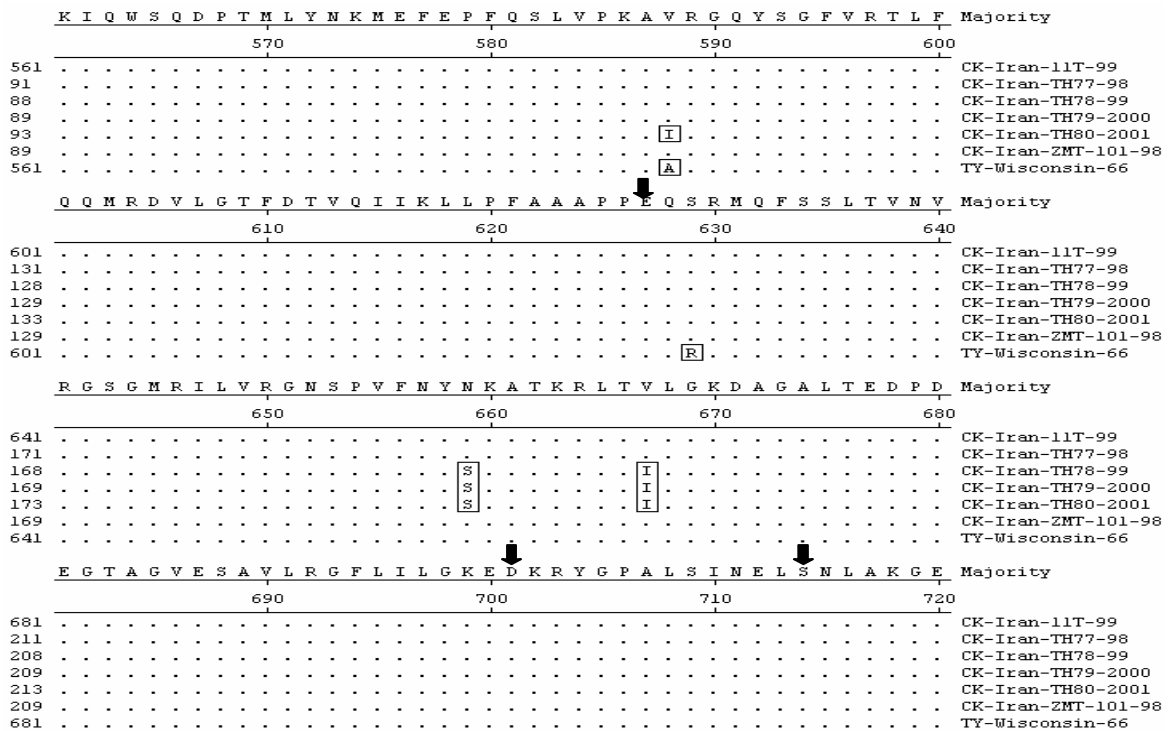


Fig. 2: Alignment of deduced amino acid sequences on PB2 genes of the five Iranian H9N2 isolates and TY/Wisconsin/66. The clear boxes show the present amino acid residue substitutions. The black arrows show the positions 627, 701 and 714.

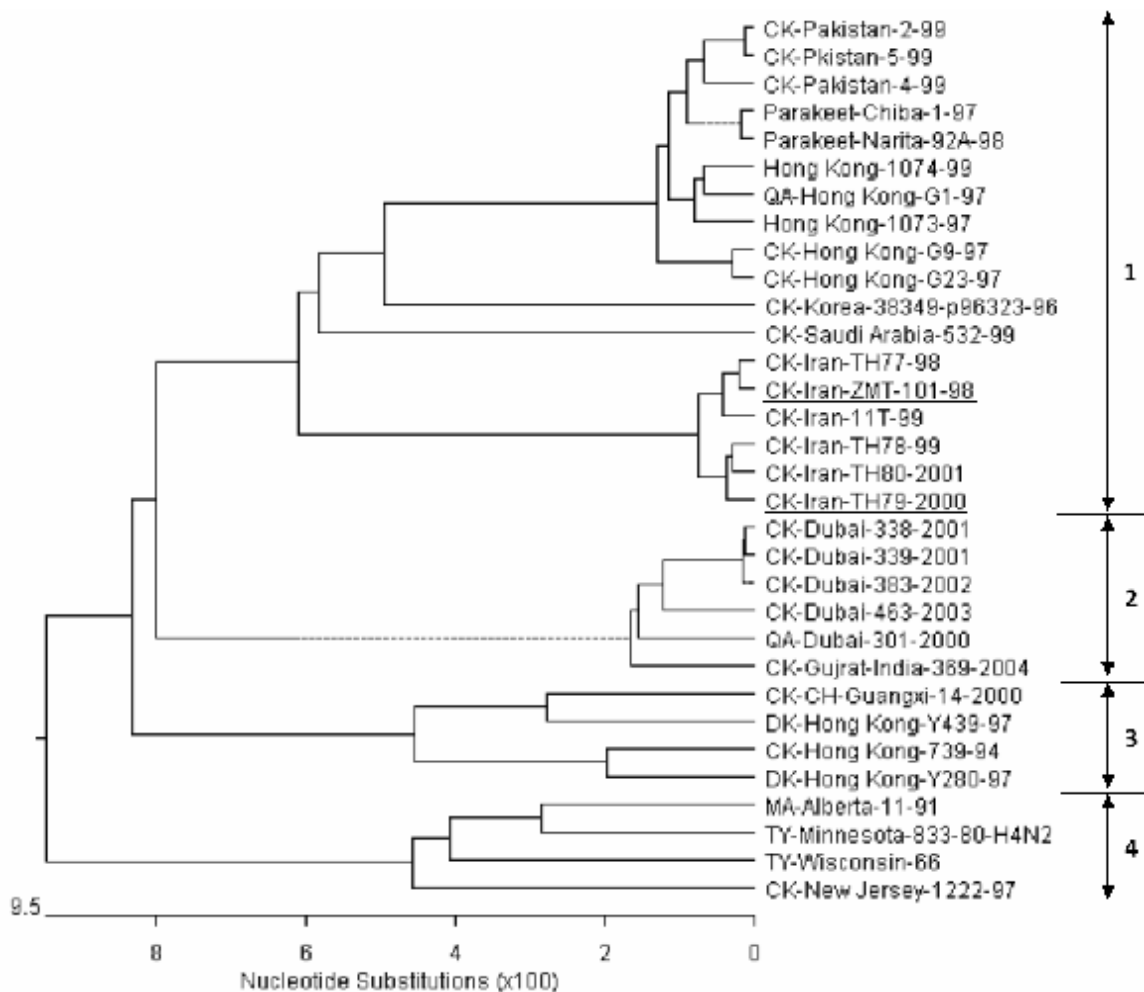


Fig. 3: Phylogenetic tree of PB2 genes of H9N2 isolates. Reconstructed by neighbor-joining method; the lengths of the horizontal lines are proportional to the minimal number of nucleotide differences required to join the nodes. Vertical lines are used for spacing branches and labels. The five isolates sequenced in the present study are shown underlined and remaining sequences are taken from GenBank. CH: China, DK: Duck, MA: Mallard, PA: Parakeet, and QA: Quail

shown that in H9N2 isolates from Tehran province of Iran from 1998 to 2001, the amino acids in positions 627, 701 and 714 are Glutamic acid-(E), Aspartic acid-(D) and Serine-(S), respectively the same as the most of the subtypes isolated from avian species including chicken (Subbarao *et al.*, 1993; Naffakh *et al.*, 2000; Labadie *et al.*, 2007). Chen *et al.* (2006) conducted a comprehensive study to validate amino acid signatures on influenza A viral genome. Among hundreds of sequences at position 627 of PB2, they have indicated the presence of Glutamic acid-(E) in 196 and Lysine-(K) in 19 sequences as avian residue, but Lysine-(K) in 838, Arginine-(R) in 2 and Isoleucine-(I) in one sequence as human residue (Chen *et al.*, 2006). The mutations

PB2 701 Asparagine-(N) and PB2 714 Arginine-(R) were shown to enhance the polymerase activity and to increase virulence in mice (Naffakh *et al.*, 2000; Hatta *et al.*, 2001; Gabriel *et al.*, 2005). Li *et al.* (2005a) showed that in 27 H9N2 isolates from domestic poultry in mainland China used in their study, there is Glutamic acid-(E) in position 627 and Aspartic acid-(D) in 701 although some of them replicated in mouse lungs and caused weight loss (Li *et al.*, 2005a). In other works on H9N2 isolates from 2007 to 2009 from the live poultry market in Shanghai and chickens in northern china the amino acid residue in position 627 and 701 of PB2 are respectively Glutamic acid-(E) and Aspartic acid-(D) (Ge *et al.*, 2009; Bi *et al.*, 2010).

The amino acid at position 588 in the five Iranian isolates is Valine-(V) but in one case (CK/Iran/TH80/2001) it is Isoleucine-(I) (Fig. 2). In position 588, defined as a genetic signature, the avian residues have been Threonine-(T) in 210, Alanine-(A) in 3 or Valine-(V) in 6 sequences and the human residues have been Isoleucine-(I) in 835, Valine-(V) in 3 or Alanine-(A) in 2 sequences (Chen *et al.*, 2006). Actually there is no report about the importance and effect of amino acid residue 659 of PB2 and its mutations. There is amino acid Isoleucine-(I) at position 667 of PB2 in three of (Fig. 2) six isolates of Tehran province (the others have Valine-(V)). It has been reported that the presence of Isoleucine-(I) at 667 is characteristic of human viruses (Hiromoto *et al.*, 2000; Chin *et al.*, 2002). These data show that the Iranian isolate CK/Iran/TH80/2001, in both positions of 588 and 667 has Isoleucine-(I).

Some previous studies indicate that increased pathogenicity in mammalian hosts is not always linked with amino acid residues in positions 627, 701 or 714 (Li *et al.*, 2005a); so pathogenicity studies in mammalian hosts is proposed to investigate the effects of the substitutions and the probable ability of Iranian H9N2 isolates to infect or even replicate in mammals.

Phylogenetic analysis data of PB2 genes indicate that the H9N2 isolates from Iran could be classified in Eurasian lineage. Comparing the nucleotide sequence identity percentage between PB2 genes of Iranian isolates of Tehran province from 1998 to 2001 with the isolates from some Middle-East countries (Table 2) shows that their similarity is not more than 90.4% between these Iranian isolates and the isolates of 2000 to 2003 from the United Arab Emirates and not more than 90.1% with the isolate from Saudi Arabia [81.4% (with CK/Iran/TH77/98) – 90.1% (with CK/Iran/11T/99)]. These data indicate that the PB2 genes of the isolates from Tehran province are not similar to the isolates from these countries, as it has been shown that the PB2 genes of isolates of the UAE from 2000 to 2003 are not similar to the isolates from Iran and Pakistan (Aamir *et al.*, 2007). These data may suggest that the PB2 genes of the Iranian and UAE isolates are not even from

the same gene pool. As shown by Tosh *et al.* (2008), although PB2 genes of Indian and UAE isolates are related to each other and derived from the same gene pool, they differ from other H9N2 isolates. The nucleotide similarity of the sequenced segment of the PB2 gene of the five Iranian isolates with viruses of the Eurasian sub-lineages like QA/HongKong/G1/97, CK/HongKong/Y280/97 and CK/Korea/38349 is not very high (Table 3), proposing that the precursors of these viruses may have been reassorted with other subtypes than H9N2. However, the reassortment of the PB2 gene of different subtypes has been shown by others (Guan *et al.*, 1999, 2000; Hoffmann *et al.*, 2000). In the same situation, the results by Tosh *et al.* (2008) showed more similarity between the nucleotide sequences of the PB2 gene of H9N2 isolates of India with an H5N6 duck isolate from Germany (DK/Potdam/2216-4/84) than the Eurasian prototype H9N2 viruses. The similarity between the PB2 gene sequences of isolates from Pakistan, used in this study, with G1 like viruses is relatively high, like with QA/HongKong/G1/97 (97.3-97.6%) and PA/Chiba/1/97 (98.7-98.9%). The similarity between PB2 genes of Pakistani isolates and Iranian isolates is low from 85.2 to 89.3%, which indicates the Pakistani isolates cannot be the gene source for the PB2 gene of these Iranian isolates. This result does not correlate with the results of studies done on the HA gene of Iranian isolates, suggesting that the H9N2 viruses from Iran should have the same gene pool origin as the Pakistani H9N2 viruses (Banks *et al.*, 2000; Karimi *et al.*, 2004; Kianizadeh *et al.*, 2006; Toroghi and Momayez, 2006; Homayounimehr *et al.*, 2010; Moosakhani *et al.*, 2010). The homology between the nucleotide sequences of the PB2 gene of H9N2 isolates of Iran is not more than 87.6% with the Chinese isolate CK/CH/Guangxi/14/2000. More studies are needed to define the probable gene pool origin for the PB2 gene of Iranian isolates and the reassortment they may have undergone.

This study is the first to analyse the nucleotide sequences of PB2 genes of H9N2 isolates of Iran. The results indicate that PB2 genes of H9N2 isolates of Tehran province are highly similar to each other (Table 2),

which shows the fewest mutations have occurred in the gene of these isolates from 1998 to 2001. It could be because of the nature of PB2 as an internal protein with less immune pressure on it compared to surface antigens like HA (Gorman *et al.*, 1990). But, since H9N2 is now the most probable candidate for the next human pandemic and it is widely spread and circulating in Iran, it is highly recommended to study the PB2 gene of isolates in recent years and in other provinces to monitor any probable mutations in it. It is very critical to study the pathogenicity of Iranian H9N2 isolates in mammalian hosts. By monitoring these mutations the probable adaptations of Iranian H9N2 isolates to mammalian hosts can be controlled.

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