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ORIGINAL ARTICLE

Genetic diversity of early (1998) and recent (2010) avian influenza H9N2 virus strains isolated from poultry in Iran

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Abstract Infection with avian influenza H9N2 virus is widespread in the Asian poultry industry, resulting in great economic losses due to mortality and a severe decline in egg production. To obtain more-comprehensive genomic data from circulating H9N2 viruses in Iran, we sequenced the whole genomes of early (Ck/IR/ZMT-101/98) and recent (Ck/IR/EBGV-88/10) isolates of this virus in Iran. The M and NS genes of Ck/IR/EBGV-88/10 shared a high level of similarity with a highly pathogenic H7N3 virus isolated from Pakistan. The cleavage site within the HA protein of these viruses contained two different motifs, RSSR and KSSR, which are similar to those found in low-pathogenic viruses. The deduced amino acid sequence of the new isolate contained the mutation Q226L, which is a characteristic of human-type sialic acid influenza receptor binding. An analysis of the viral amino acid sequence of the M2 protein of the recent strain revealed a V27A mutation, which is associated with amantadine resistance in avian influenza virus. The present results emphasize the need for continuous surveillance of H9N2 viruses in poultry and the human population to obtain more information about the nature and evolution of future pandemic influenza viruses.

Introduction

Since its first detection in turkeys in 1966, avian influenza virus (AIV) subtype H9N2 has mainly been found in shorebirds and wild ducks [20]. In the mid-1990s, outbreaks due to H9N2 influenza viruses have occurred in Germany, Italy, Ireland, South Africa, the USA, Korea and China in multiple avian species. Since 1998, H9N2 infections have been reported from the Middle East countries, causing widespread outbreaks in commercial chickens. H9N2 infections have become endemic in several countries despite the use of wide-scale vaccination programs against H9N2 virus [2]. The first record of human infection with H9N2 was reported from Hong Kong in 1999, and since then, sporadic cases have continued to occur [22]. H9N2 virus has also been isolated in swine, raising the possibility of further transfer to humans due to reassortment [25]. These findings suggest that the pandemic potential of H9N2 virus, in addition to H5N1 virus, could become an increasing concern.

Based on epidemiological and genetic studies, there are two major gene pools of H9N2 influenza viruses worldwide: the North American and the Eurasian. Eurasian lineages are subdivided into two major sub-lineages: A/Quail/Hong Kong/G1/97 (Qa/HK/G1/97, G1) and A/Duck/Hong Kong/Y280/97 (Dk/HK/Y280/97, Y280) [40]. Recent analysis of H9N2 viruses revealed four monophyletic groups of sub-lineage G1 in Central Asia and the Middle East (A, B, C and D), and viruses from Iran are identified as group B and D [11].

The first outbreak of H9N2 virus, strain Ck/IR/ZMT-101/98, in Iran was reported in layer flocks in 1998 [38]. Despite the use of inactivated vaccine against this virus, large outbreaks continue to occur [28]. The effect of immunological pressure by the vaccine and cocirculation of H9N2 with other subtype of highly pathogenic avian influenza (HPAI) such as H5 and H7 in wild birds in Iran can increase the probability of novel variant and reassortant viruses [10]. To our knowledge, little is known about the gene constellation of the H9N2 influenza viruses that are

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circulating in Iran; hence, we have extended this information by sequencing eight full-length segments of Ck/IR/ZMT-101/98 (IR-98) and Ck/IR/EBGV-88/10 (IR-10) viruses. Furthermore, we conducted phylogenetic analysis of these viruses to compare their relationship to the other AIVs.

Materials and methods

Viruses

IR-98 and IR-10 viruses were obtained from the Faculty of Veterinary Medicine, University of Tehran. IR-98 was isolated from layer flocks in 1998 in Tehran Province and was also used as an inactivated vaccine seed since the first isolation of H9N2 virus in Iran. IR-10 was originally isolated from broiler flocks suffering from severe respiratory signs and high mortality. All isolates were propagated in the allantoic cavities of ten-day-old pathogen-free chicken eggs to obtain a virus stock. All allantoic fluids were confirmed by standard hemagglutination inhibition assay and kept at −70 °C for further use [30].

RNA extraction

RNA was extracted from infectious allantoic fluids using RNX^{TM}-plus solution (Cinnagen, Iran) following manufacturer's instructions. The purity of the extracted RNA was determined by calculating the ratio of the readings at 260 and 280 nm.

Reverse transcription (RT) polymerase chain reaction (PCR)

RT was carried out by using a RevertAid^{TM} first-strand cDNA synthesis kit (Fermentas, Canada) and Uni12 primer, 5′-AGCAAAAGCAGG-3′ [15]. The subsequent PCRs were performed using universal primers as described previously [15], except for the HA gene, which was amplified as two overlapping amplicons using a series of primers (primer sequences available on request). PCR products were purified using a GeneJET^{TM} gel extraction kit (Fermentas, Canada). PCR-purified products were cloned using a ClonJET^{TM} PCR cloning kit (Fermentas, Canada).

Sequencing and phylogenetic analysis

DNA sequencing of cloned products was performed in both directions by a DNA service company (Source BioScience Co., UK). Internal primers were designed for each of the polymerase genes (primer sequences available on request). All sequences were assembled and translated by using BioEdit Package, version 7.1.3.0 [13]. BLAST analysis was initially performed to retrieve sequences related to our queries from the GenBank databases to compare them with all eight genome segments determined in this study. Multiple sequence alignments of nucleotide and deduced amino acid sequences were performed for the eight full-length genes of the query strains using the CLUSTAL W method in the Molecular Evolutionary Genetics Analysis (MEGA) software, version 5.0 [37]. Unrooted phylogenetic trees were constructed for each gene of two strains by the neighbor-joining method. The robustness of the phylogenetic analysis and significance of the branch order were evaluated by bootstrapping with 1000 replicates.

Nucleotide sequence accession numbers

The nucleotide sequences of all eight gene segments of IR-98 and IR-10 have been deposited in the GenBank database under accession numbers EF044307, JN646748, JQ364984, JQ364985 and JX465619 to JX465630.

Results

Homology analysis

All eight full-length genes of two Iranian H9N2 viruses were sequenced and aligned with different sequences from H9N2 viruses listed in GenBank. Genetic comparisons of the nucleotide and deduced amino acid sequences of eight genes between the two isolates of H9N2 in Iran revealed sequence identity ranging from 89.2 % to 95.4 % and 93.5 % to 98.0 %, respectively. The homology of the surface glycoprotein genes H9 and N2 reached to 91.7 % and 92.9 %, respectively, among these viruses at the nucleotide level. The lowest nucleotide sequence identity was found in the PB2 gene (89.2 %), while the M gene showed less diversity (95.4 %) than the other internal genes.

The nucleotide sequence similarities of the IR-98 and IR-10 genes are shown in Table 1. The HA, NA, and M genes of IR-98 virus displayed the highest nucleotide sequence identities to a Japanese isolate (98.5-99.5 %). The ribonucleoprotein (RNP) complex genes (PB2, PB1, PA and NP) of this isolate were most homologous to those of other subtypes, such as H5N2, H6N1 and H5N9. The PB2 and PB1 genes of the IR-98 isolate showed a close relationship to those of an H5N2 duck isolate from Germany (Dk/PO/1402-61/86; 95.6 % and 96.6 %, respectively). The PA and NP genes were 96.6 % and 97.9 % similar to those of Ck/TW/0824/97 (H6N1) and Ck/IT/9097/97 (H5N9), respectively. The nucleotide sequence of the NS gene of the IR-98 strain was related to those of

Table 1 Nucleotide sequence homology of eight genes between Iranian isolates and other H9N2 viruses

Virus [A]	Gene	Most similar to	Homology (%)
Ck/IR/ZMT-101/98	PB2	Dk/PO/1402-61/86 (H5N2)	95.6[B]
		Ty/EN/50-92/91 (H5N1)	95.1
	PB1	Dk/PO/1402-61/86 (H5N2)	96.6
		MDk/AS/266/82 (H14N5)	96.2
	PA	Ck/TW/0824/97 (H6N1)	96.6
		Dk/AL/1285/91 (H5N3)	96.4
	HA	Pa/CH/1/97 (H9N2)	98.5
		HK/1074/97 (H9N2)	96.9
	NP	Ck/IT/9097/97(H5N9)	97.9
		Dk/HK/Y439/97 (H9N2)	97.3
	NA	Pa/CH/1/97 (H9N2)	99.3
		HK/1047/97 (H9N2)	97.4
	M	Pa/CH/1/97 (H9N2)	99.5
		HK/483/97 (H5N1)	99.3
	NS	Dk/HK/P54/92 (H11N9)	97.9
		Dk/HK/P50/97 (H11N9)	97.7
Ck/IR/EBGV-88/10	PB2	Ck/PK/UDL-01/06 (H9N2)	97.0
		Ck/PK/UDL-02/06 (H9N2)	96.7
	PB1	Ck/PK/UDL-01/06 (H9N2)	98.1
		Ck/PK/UDL-02/05 (H9N2)	97.0
	PA	Ck/PK/UDL-01/08 (H9N2)	97.2
		Ck/PK/UDL-03/07 (H9N2)	97.2
	HA	Ck/PK/UDL-01/08 (H9N2)	97.7
		Ck/PK/UDL-03/07 (H9N2)	97.7
	NP	Ck/PK/UDL-03/07 (H9N2)	98.5
		Ck/PK/UDL-03/08 (H9N2)	98.3
	NA	Ck/PK/UDL-01/06 (H9N2)	97.0
		Ck/PK/UDL-03/07 (H9N2)	96.1
	M	Ck/PK/NARC-100/04 (H7N3)	96.9
		Ck/PK/UDL-01/05 (H9N2)	96.6
	NS	Ck/PK/UDL-02/06 (H9N2)	98.6
		Ck/PK/NARC-100/04 (H7N3)	96.1

[A] Abbreviations: Ck, chicken; DK, duck; Qa, quail; Pa, parakeet; MDk, mallard duck; Ty, turkey; AE, Arabian Emirates; AL, Altai; AS, Astrakhan; BJ, Beijing; CH, Chiba; DU, Dubai; EN, England; GD, Guangdong; HK, Hong Kong; IL, Israel; IN, India; IQ, Iraq; IR, Iran; IT, Italy; JO, Jordan; KR, Korea; KW, Kuwait; LB, Lebanon; NC, Nanchang; PK, Pakistan; PO, Potsdam; SA, Saudi Arabia; SH, Shanghai; TW, Taiwan; WI, Wisconsin

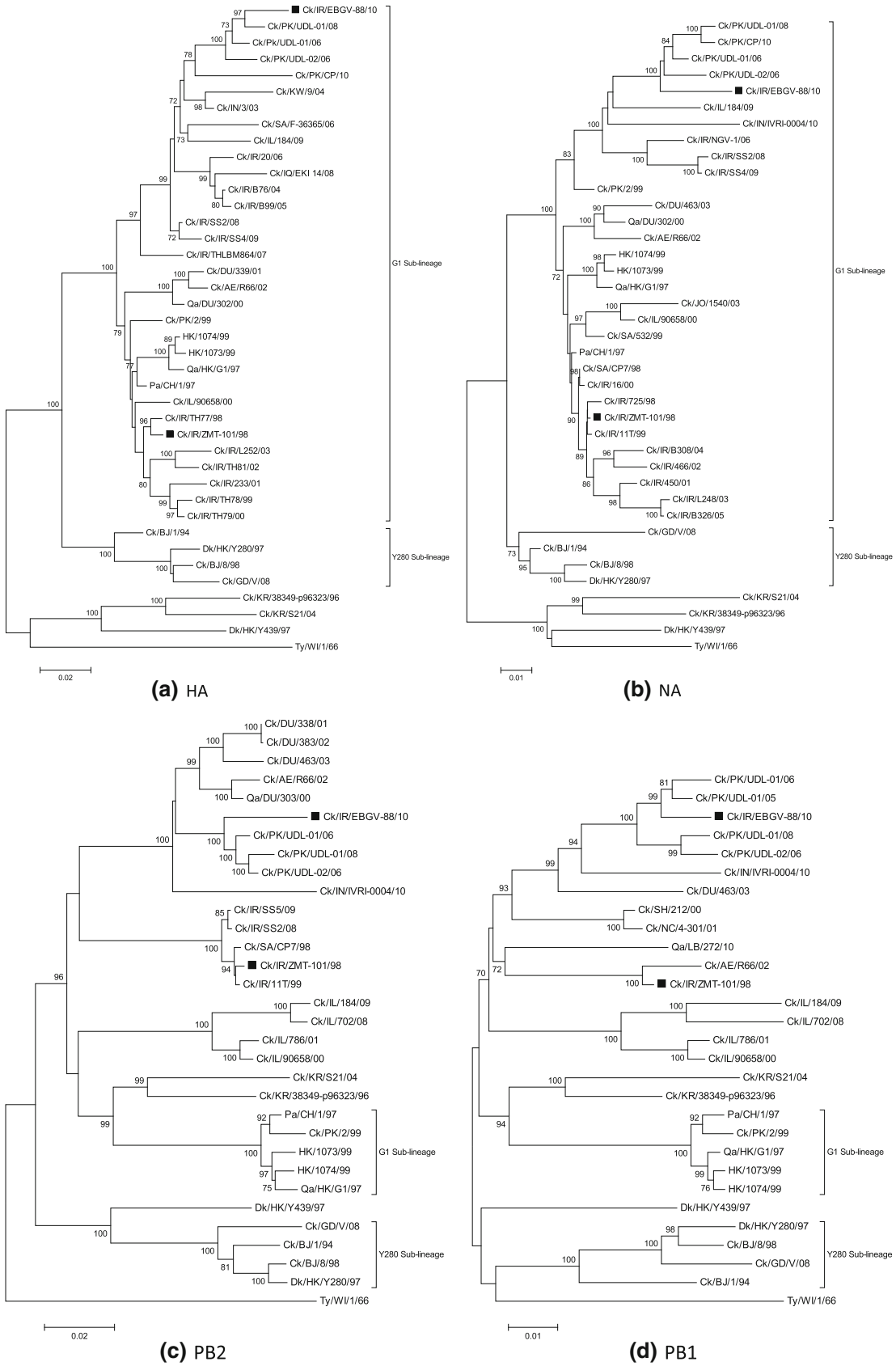
[B] Homology were calculated based on complete open reading frames of PB2 (28-2307), PB1 (25-2298), PA (25-2175), HA (34-1716), NP (46-1542), NA (20-1429), M (26-1007) and NS (27-864)

H11N9 duck isolates from Hong Kong, Dk/HK/P54/92 (97.7 %) and Dk/HK/P50/97 (97.9 %).

Sequence analysis of the complete genome indicated that the IR-10 strain is closely related to H9N2 strains isolated from Pakistan between 2005 and 2008 (96.1-98.6 %). Interestingly, an Iranian H9N2 virus isolated in 2010 had a close relationship in the nucleotide sequences of M and NS genes to an H7N3 virus (Ck/PK/NARC-100/04; 96.9 % and 96.1 %, respectively) isolated in Pakistan, which shares a border with Iran. This homology may indicate a possible reassortment between low-pathogenic H9N2 from Iran and highly pathogenic H7N3 from Pakistan.

Phylogenetic analysis

The phylogenetic tree of all eight nucleotide sequences of Iranian AIVs is shown in Fig. 1. The results for surface glycoprotein genes (H9 and N2 genes) revealed that both Iranian isolates clustered into the G1 sub-lineage, together


Ck/IR/EBGV-88/10
Ck/PK/UDL-01/08
Ck/Pk/UDL-01/06
Ck/PK/UDL-02/06
Ck/PK/CP/10
Ck/KW/9/04
Ck/IN/3/03
Ck/SA/F-36365/06
Ck/IL/184/09
Ck/IR/20/06
Ck/IQ/EKI 14/08
Ck/IR/B76/04
Ck/IR/B99/05
Ck/IR/SS2/08
Ck/IR/SS4/09
Ck/IR/THLBM864/07
Ck/DU/339/01
Ck/AE/R66/02
Qa/DU/302/00
Ck/PK/2/99
HK/1074/99
HK/1073/99
Qa/HK/G1/97
Pa/CH/1/97
Ck/IL/90658/00
Ck/IR/TH77/98
Ck/IR/ZMT-101/98
Ck/IR/L252/03
Ck/IR/TH81/02
Ck/IR/233/01
Ck/IR/TH78/99
Ck/IR/TH79/00
G1 Sub-lineage
Ck/BJ/1/94
Dk/HK/Y280/97
Ck/BJ/8/98
Ck/GD/V/08
Y280 Sub-lineage
Ck/KR/38349-p96323/96
Ck/KR/S21/04
Dk/HK/Y439/97
Ty/WI/1/66
0.02
(a) HA
Ck/PK/UDL-01/08
Ck/PK/CP/10
Ck/PK/UDL-01/06
Ck/PK/UDL-02/06
Ck/IR/EBGV-88/10
Ck/IL/184/09
Ck/IN/IVRI-0004/10
Ck/IR/NGV-1/06
Ck/IR/SS2/08
Ck/IR/SS4/09
Ck/PK/2/99
Ck/DU/463/03
Qa/DU/302/00
Ck/AE/R66/02
HK/1074/99
HK/1073/99
Qa/HK/G1/97
Ck/JO/1540/03
Ck/IL/90658/00
Ck/SA/532/99
Pa/CH/1/97
Ck/SA/CP7/98
Ck/IR/16/00
Ck/IR/725/98
Ck/IR/ZMT-101/98
Ck/IR/11T/99
Ck/IR/B308/04
Ck/IR/466/02
Ck/IR/450/01
Ck/IR/L248/03
Ck/IR/B326/05
G1 Sub-lineage
Ck/GD/V/08
Ck/BJ/1/94
Ck/BJ/8/98
Dk/HK/Y280/97
Y280 Sub-lineage
Ck/KR/S21/04
Ck/KR/38349-p96323/96
Dk/HK/Y439/97
Ty/WI/1/66
0.01
(b) NA
Ck/DU/338/01
Ck/DU/383/02
Ck/DU/463/03
Ck/AE/R66/02
Qa/DU/303/00
Ck/IR/EBGV-88/10
Ck/PK/UDL-01/06
Ck/PK/UDL-01/08
Ck/PK/UDL-02/06
Ck/IN/IVRI-0004/10
Ck/IR/SS5/09
Ck/IR/SS2/08
Ck/SA/CP7/98
Ck/IR/ZMT-101/98
Ck/IR/11T/99
Ck/IL/184/09
Ck/IL/702/08
Ck/IL/786/01
Ck/IL/90658/00
Ck/KR/S21/04
Ck/KR/38349-p96323/96
Pa/CH/1/97
Ck/PK/2/99
HK/1073/99
HK/1074/99
Qa/HK/G1/97
G1 Sub-lineage
Dk/HK/Y439/97
Ck/GD/V/08
Ck/BJ/1/94
Ck/BJ/8/98
Dk/HK/Y280/97
Y280 Sub-lineage
Ty/WI/1/66
0.02
(c) PB2
Ck/PK/UDL-01/06
Ck/PK/UDL-01/05
Ck/IR/EBGV-88/10
Ck/PK/UDL-01/08
Ck/PK/UDL-02/06
Ck/IN/IVRI-0004/10
Ck/DU/463/03
Ck/SH/212/00
Ck/NC/4-301/01
Qa/LB/272/10
Ck/AE/R66/02
Ck/IR/ZMT-101/98
Ck/IL/184/09
Ck/IL/702/08
Ck/IL/786/01
Ck/IL/90658/00
Ck/KR/S21/04
Ck/KR/38349-p96323/96
Pa/CH/1/97
Ck/PK/2/99
Qa/HK/G1/97
HK/1073/99
HK/1074/99
G1 Sub-lineage
Dk/HK/Y439/97
Dk/HK/Y280/97
Ck/BJ/8/98
Ck/GD/V/08
Ck/BJ/1/94
Y280 Sub-lineage
Ty/WI/1/66
0.01
(d) PB1

◀**Fig. 1** Phylogenetic relationships of (a) HA, (b) NA, (c) PB2, (d) PB1, (e) PA, (f) NP, (g) M and (f) NS genes of H9N2 avian influenza viruses. The isolates that were analyzed are marked with *squares*, and a *triangle* indicates H7N3 virus. Phylogenetic trees were generated by the distance-based neighbor-joining method using MEGA software, version 5.0. The analysis was based on the following nucleotides: PB2 (53-2289), PB1 (46-2230), PA (25-2166), HA (34-1599), NP (46-1491), NA (20-1412), M (26-1001) and NS (30-859). Vertical lines indicate G1 and Y280 sub-lineages. The scale bars represent the number of nucleotide substitutions per site. Bootstrap support values ≥70 % are shown above the branches. Abbreviations are listed in Table 1

with all of the Iranian and Middle Eastern viruses that were obtained from GenBank in this present study. In the case of H9, IR-10 shared a common ancestor with Pakistani isolates, whereas IR-98 fell into another cluster with other Iranian strains that were isolated before 2003. Phylogenetic analysis of the NA gene showed an evolutionary pattern similar to that of the HA gene, in which new strains isolated during 2006-2009 clustered into a common group along with IR-10.

Based on phylogenetic analysis, the internal genes of both isolates had more diverse sources than the surface genes, which belong to unknown sub-lineages, except for the M genes of both isolates and the NP gene of IR-10 virus, which formed a clade similar to those of the surface glycoproteins. The PB2, PB1 and PA genes of IR-10 fell into a group with Pakistani isolates, while IR-98 were placed in a different group together with other Middle Eastern viruses. The NP gene phylogenetic tree revealed that IR-10 belongs to the G1 sub-lineage, but IR-98 and another Iranian isolates were placed in another group closer to the Saudi Arabian strain Ck/SA/CP7/98. It was also noted that Dk/HK/Y439/97 shared an outgroup relationship with IR-98 in the NP gene tree, suggesting that these viruses were derived from same gene pool. In the NS gene phylogenetic tree, IR-10 clustered in a different group with IR-98, together with other Iranian and Pakistani isolates. The M and NS genes of IR-10 clustered into groups closer to the H7N3 virus Ck/PK/NARC-100/04. Taken together, our finding revealed that there has possibly been genetic reassortment between IR-10 and other subtypes of HPAI, such as H7N3.

Genetic characteristics

All of the deduced amino acid sequences of eight full-length genes of two Iranian strains were determined and compared with those of other representative isolates from the GenBank database (Tables 2, 3).

Hemagglutinin

The deduced amino acid sequences of the HA protein indicated that both isolates exhibited different motifs at the cleavage site. The first Iranian isolate IR-98 possessed an RSSR motif, while IR-10 had a substitution at residue 326 (R→K) (H3 numbering hereafter), which is similar to other Middle Eastern and Asian isolates. This is the first published report of a new KSSR motif at the connecting peptide of Iranian isolates. Both isolates had seven potential glycosylation sites with an N-X-S/T motif (where X can be any amino acid except proline), five of which were in the HA1 portion, and two of which were in HA2 part of the molecule.

Residues at positions 98, 153, 155, 183, 190, 226, 227 and 228 are major components of the receptor-binding site of the HA molecule. Examination of amino acid sequences at these receptor binding sites revealed that both isolates had conserved residues at positions 98, 153, 155, 183 and 228. In IR-10, four residues at position 190, which is in the receptor-binding site; positions 226 and 227, which are at the left edge of receptor-binding site; and position 138, which is at the right edge of the receptor-binding site, displayed E190T, Q226L, Q227I and A138S variations, respectively, as compared with IR-98. An amino acid change from glutamine to leucine at position 226 is a characteristic of human-type sialic acid influenza receptor binding.

Neuraminidase

The neuraminidase stalk region of these two isolates did not have a deletion at positions 38 and 39 like the two prototype viruses Qa/HK/G1/97 and HK/1073/97. Deletions of three amino acid residues at positions 63, 64 and 65 in the stalk region of Dk/HK/Y280/97 were also not observed in these isolates.

Analysis of potential glycosylation sites in the NA gene of the two Iranian isolates revealed seven conserved sites in the full length of the NA gene. The positions were located at 61, 69, 86, 146, 200, 234 and 402; however, both isolates contained additional potential glycosylation sites at different positions – residue 70 in IR-98 and residue 44 in IR-10 – due to the substitution of G70N and P45S, respectively.

Comparison of three separate loops of the hemadsorbing (HB) sites of the two isolates revealed that IR-10 had one amino acid substitution at position 403 that was similar to that found in Pakistani isolates. Among the two Iranian isolates, no mutations were observed in the active-site residues, which are associated with resistance to neuraminidase inhibitor drugs (oseltamivir).

Genetic characteristic of internal proteins

The deduced amino acid sequences of the RNP complex genes were aligned and compared with reference H9N2 viruses. Numerous substitutions within the polymerase complex and NP are known to alter host range and

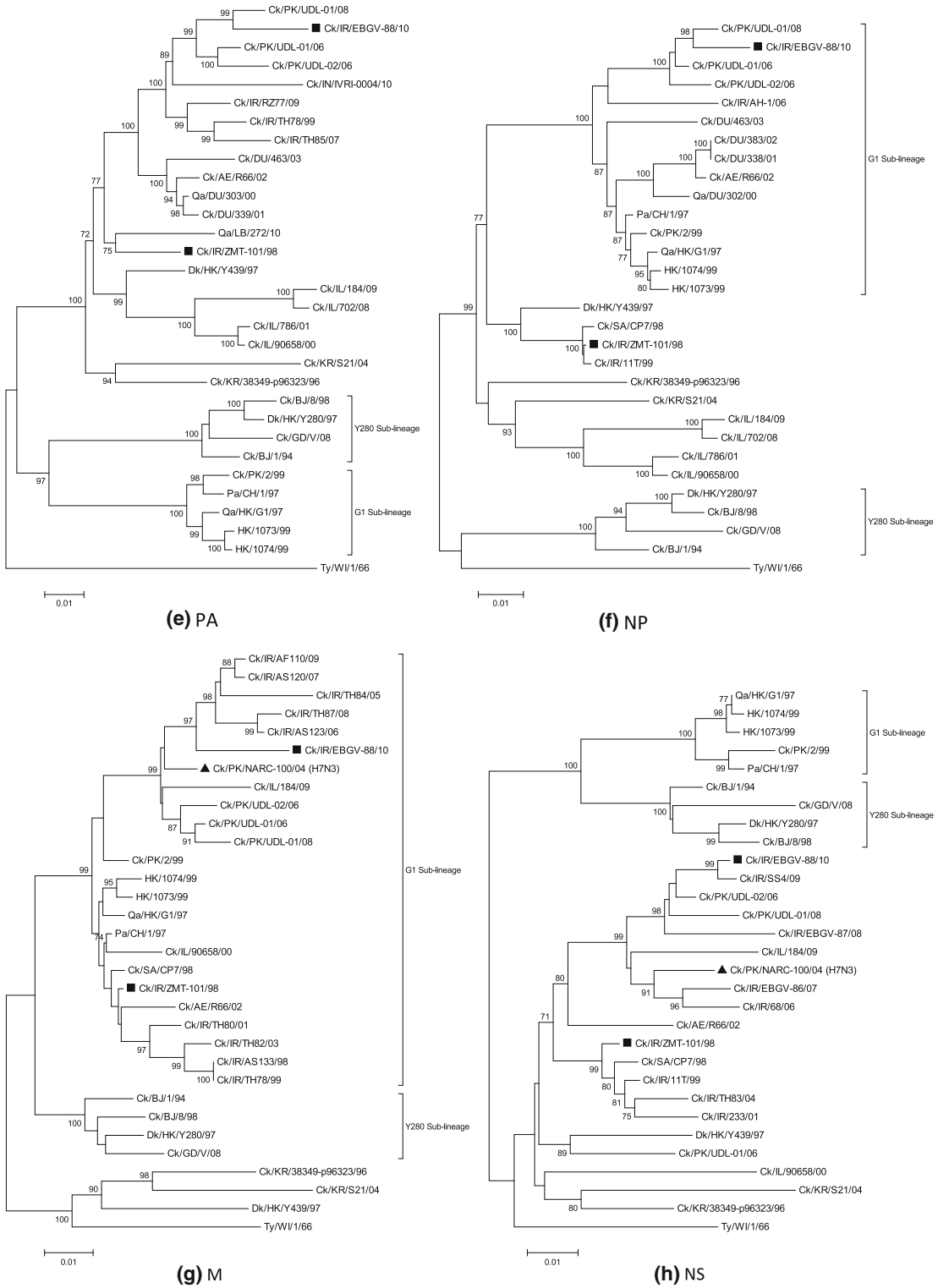


Fig. 1 continued

virulence [7, 27, 34]. Analysis of amino acids associated with these determinants revealed that the two tested strains had most of the avian host signatures except position 356 in the PA protein (L→R in IR-10) and residues 136 (L→M in IR-98) and 372 (D→E in IR-10) in the NP protein, which were changed to human amino acid signature.

The current Iranian strains contain glutamic acid at position 627 in the PB2 protein. Therefore, a single E627K

Table 2 Comparison of amino acid sequences of Iranian HA proteins with those of other H9N2 viruses

Virus [B]	HA[A]											
	Receptor-binding site							Left edge of receptor-binding pocket	Right edge of receptor-binding pocket	Potential glycosylation site		Cleavage site
	98	153	155	183	190	194	195	224-229	134-138	HA1	HA2	326-329
Ck/IR/ZMT-101/98	P	W	T	H	E	L	Y	NGQQGR	GTSKA	21, 94, 128, 289, 296	154, 213	RSSR
Ck/IR/EBGV-88/10	P	W	T	H	T	L	Y	NGLIGR	GTSKS	21, 94, 128, 289, 296	154, 213	KSSR
Ck/AE/R66/02	P	W	T	H	E	L	Y	NGQLGR	GTSKA	21, 94, 128, 210, 289, 296	154, 213	RSSR
Ck/IL/184/09	P	W	T	H	A	L	Y	NGLIGR	GTSKS	21, 94, 128, 289, 296	154, 213	RSSR
Ck/PK/2/99	P	W	T	H	A	L	Y	NGQQGR	GTSKA	21, 94, 128, 210, 289, 296	154, 213	RSSR
Ck/PK/UDL-01/06	P	W	T	H	A	L	Y	NGLIGR	GTSKS	21, 94, 128, 289, 296	154, 213	RSSR
Ck/PK/UDL-02/06	P	W	T	H	A	L	Y	NGLIGR	GTSKS	21, 94, 128, 289, 296	154, 213	RSSR
Ck/PK/UDL-01/08	P	W	T	H	A	L	Y	NGLIGR	GTSKS	21, 94, 128, 289, 296	154, 213	KSSR
HK/1073/99	P	W	T	H	E	L	Y	NGLQGR	GISRA	21, 94, 128, 198, 210, 289, 296	154, 213	RSSR
Qa/HK/G1/97	P	W	T	H	E	L	Y	NDLQGR	GTSRA	21, 94, 128, 198, 210, 289, 296	154	RSSR
Dk/HK/Y280/97	P	W	T	N	T	L	Y	NGLQGR	GTSKA	21, 128, 210, 289, 296	154	RSSR
Pa/CH/1/97	P	W	T	H	E	L	Y	NGLQGR	GTSKA	21, 94, 128, 210, 289, 296	154, 213	RSSR
Ty/WI/1/66	P	W	T	H	E	L	Y	NGQQGR	GTSRA	21, 128, 210, 289, 296	154, 213	VSSR

[A] Amino acid numbering is based on the HA sequence of the human H3 subtype

[B] Abbreviations are listed in Table 1

mutation at this position enhances pathogenicity and adaptation to mammalian hosts. A PB2 D701N mutation is also associated with influenza virus virulence in mammals; however, the PB2 proteins of examined strains did not contain this mutation. Viruses with an N66S mutation in the PB1-F2 protein had increased virulence; however, our isolates did not show this mutation.

Sequence analysis of the M2 protein revealed that IR-10 displayed the V27A mutation, suggesting that this virus has resistance to inhibitors of the viral M2 protein, such as amantadine. The NS1 protein of our isolates possessed a PDZ ligand (PL) motif at the C-terminus. The PL motif of both isolates sequenced in this study contained ESEV (IR-98) and KSEI (IR-10) motifs. Most AIVs possess an ESEV motif at the very end of NS1 protein; however, the KSEI motif is rare and similar to those found recently in Pakistani isolates.

Discussion

Since the first outbreak of H9N2 AIV in Iran in the late 1990s, H9N2 avian influenza has become geographically widespread across the country, which has resulted in the wide use of vaccination against this subtype to control the disease. To date, despite extensive use of inactivated vaccine, endemic outbreaks of H9N2 avian influenza have often occurred and caused serious economic losses throughout the country, particularly in broiler chickens. Because influenza virus mutates rapidly, vaccine strains

Table 3 Comparison of amino acid sequences of Iranian NA, PB2, PB1-F2, PA, NP, M2 and NS1 proteins with those of other H9N2 viruses

Virus [A]	NA					PB2		PB1-F2	PA	NP		M2					NS1
	Stalk deletion	Hemadsorbing site			Potential glycosylation site	627	701	66	356	136	372	26	27	30	31	34	PDZ ligand motif
		366-373	399-404	431-433													
Ck/IR/ZMT-101/98	-	IKKDSRAG	DSDNLS	PQE	61, 69, 70, 86, 146, 200, 234, 402	E	D	N	K	M	E	L	V	A	S	G	ESEV
Ck/IR/EBGV-88/10	-	IKKDSRAG	DSDNRS	PQE	44, 61, 69, 86, 146, 200, 234, 402	E	D	N	R	I	D	L	A	A	S	G	KSEI
Ck/AE/R66/02	-	IKEDLRAG	DSDNWS	PQE	61, 69, 70, 86, 146, 200, 234, 402	E	D	N	K	M	E	L	V	A	S	G	KQKR
Ck/IL/184/09	-	IKKDSRAG	DSDNWS	PQE	44, 61, 69, 86, 146, 200, 234, 402	V	D	S	K	L	E	L	V	A	S	G	KSEV
Ck/PK/2/99	-	IKKDSRTG	DSDNWS	PQE	61, 69, 86, 146, 200, 234, 402	E	D	N	K	M	E	L	V	A	S	G	EPEV
Ck/PK/UDL-01/06	-	IKKDSRAG	DSDNRS	PQE	44, 61, 69, 86, 146, 200, 234, 402	E	D	N	K	I	D	L	V	A	S	G	LPPK
Ck/PK/UDL-02/06	-	IKKDSRAG	DSDNRS	PQE	44, 61, 69, 86, 146, 200, 402	E	D	N	K	I	D	L	V	A	S	G	KSEI
Ck/PK/UDL-01/08	-	IKKDSRAG	DSDNRS	PQE	44, 61, 69, 86, 146, 200, 234, 402	E	D	N	K	I	D	L	V	A	S	G	KSEI
HK/1073/99	38-39	IKKDSRSG	DSDNWS	PQE	59, 67, 68, 84, 144, 198, 232, 400	E	D	N	K	M	E	L	V	A	S	G	EPEV
Qa/HK/G1/97	38-39	IKKDSRSG	DSDIRS	PQE	59, 67, 68, 84, 144, 198, 232	E	D	N	K	M	E	L	V	A	S	G	EPEV
Dk/HK/Y280/97	63-65	IKEDSRSG	DSDNWS	PQE	66, 83, 143, 197, 231, 399	E	D	N	K	L	E	L	V	A	S	G	EPEV
Pa/CH/1/97	-	IKKDSRAG	DSDNWS	PQE	61, 69, 70, 86, 146, 200, 234, 402	E	D	N	K	M	E	L	V	A	S	G	EPEV
Ty/WI/1/66	-	ISKDSRSG	DSNNWS	PQE	61, 70, 86, 146, 200, 234, 402	E	D	D	K	L	E	L	V	A	S	G	ESEV

[A] Abbreviations are listed in Table 1

need to be changed to match the circulating virus in order to remain effective [33]. In Iran, inactivated vaccines derived from viruses isolated in the late 1990s have been extensively applied in chicken flocks to control disease [9, 39]. This study aimed at determining the full genetic relationship and evolution of H9N2 influenza virus after 10 years in Iran. More specifically, IR-98 virus isolated during the first epidemic in Iran and the recently isolated virus IR-10 were completely sequenced and compared to the H9N2 sequence data available in GenBank.

Nucleotide sequence analysis of HA genes of strains reported in the current study demonstrated that the IR-10 strain shared only 91.7 % sequence identity with IR-98. Because of the broad use of vaccines derived from earlier isolates after the first outbreak in Iran and primary protection by HA, the vaccine strain must match the HA of circulating virus in the field. A closer similarity between the HA sequences of vaccine and field viruses leads to significant reduction in challenge virus replication and viral shedding from the respiratory tract [36]. In the phylogenetic tree of the surface glycoproteins, Iranian isolate IR-10, along with the Pakistani isolates, formed a cluster with high nucleotide sequence homology, whereas IR-98 strain fell into a different cluster with H9N2 isolated from Japan and other Middle East countries, similar to those isolated from the United Arab Emirates [1]. The importation of wild psittacine birds from Pakistan as pets is thought to be the cause of introduction of this virus into Japan [23]. Furthermore, it has been reported that the HA genes of viruses isolated from Germany, Iran, Pakistan and Saudi Arabia during 1998 and 1999 were closely related to strain Qa/HK/G1/97, which shared internal genes similar to those of H5N1 and H9N2 strains responsible for human infection in Hong Kong [6].

Based on a recent extensive phylogenetic analysis of H9N2 viruses in central Asia and the Middle East, there are four distinct and cocirculating groups (A, B, C and D). Viruses isolated from Iran form two clusters: B and D. Group B contains viruses from Pakistan and Afghanistan as well as Iranian viruses isolated during 2003-2009; however, group D consists only of viruses from Iran that circulated from 1998-2007. HA, NA and M genome segments of all groups, as well as the NP gene of group B, share the same progenitor (G1 sub-lineage), while the other genes did not show any relationship to those of the G1 and Y280 sub-lineages, generating many different genotypes. Viruses isolated from Pakistan were the main source of all genes for group B [11]. The results of the current study indicate that IR-10 belongs to monophyletic group B, while IR-98 belongs to group D. Whether this difference between clusters of tested isolates in the last 10 years is due to movement of poultry or to some extent other means of transmission, such as wild birds, is not known [11].

It is noteworthy that the M and NS genes of the new Iranian strain IR-10 shared high homology with that of an HPAI virus isolated from a chicken, Ck/PK/NARC-100/04 (H7N3) suggesting a common ancestral origin of these genes. One of the mechanisms by which AIVs evolve is reassortment, and several studies have identified reassortment between H9N2 and the HPAI H7N3 and H5N1 viruses [8, 18]. Whether IR-10 donates these genes to H7N3 or the exchange was in the opposite direction is not known.

The primary virulence factor of avian influenza virus is the structure of the cleavage site of HA gene [4]. The deduced amino acid sequences of the HA cleavage sites of the viruses analyzed revealed that IR-98 had an RSSR motif, similar to the cleavage sites of most H9N2 AIVs in chickens [1, 16]; however, IR-10 had a KSSR motif, which has been rarely seen in H9N2 viruses [18, 41]. Although both amino acids at the -4 position of the cleavage site are basic amino acids, it is not known whether this substitution can alter the virulence of H9N2. The absence of multiple basic amino acids at the cleavage sites of the isolates studied here suggests the low-pathogenic nature of these viruses; however, only one additional nucleotide substitution at the -2 position results in an increase in the number of basic amino acids in the cleavage motif (R/K-X-R-R), which is a characteristic of HPAI virus [31].

Changes in amino acid residues in the receptor-binding region of HA can cause alteration of influenza binding specificity. Among the seven amino acid residues of HA1 involved in the receptor-binding site, six of them showed conservation, while the IR-10 isolate had threonine, in comparison to glutamic acid in the IR-98 isolate, at position 190. HAs of avian viruses with E190 favor a sialic acid α-2,3 galactose linkage, which is their preferred receptor, while substitution of E190V, E190T and E190A results in high, intermediate and low binding affinity, respectively, to human-like receptors with a sialic acid α-2,6 galactose linkage [25]. Other amino acid residues at the left and right edge of the binding pocket are associated with receptor binding sites containing ^{224}NGQ/LQ/IGR^{229} and ^{134}GTSKA/S^{138} motifs, respectively. In these motifs, three mutations in Q226L, Q227I and A138S were observed in the IR-10 strain when compared to IR-98. A change in receptor preference from birds to mammals requires a single Q226L mutation. Another change of G228 to S228 would also greatly increase virus binding to the human receptor in H2 and H3 [32]. The effect of the Q227I substitution on the receptor-binding specificity of IR-10 is not known and requires detailed structural analysis to determine its significance. A substitution of alanine to serine at position 138 in the receptor-binding pocket in H1 has been reported to increase binding to a sialic acid

α-2,6 galactose linkage and a loss of the ability to bind avian receptors [24].

Analysis of potential glycosylation sites (N-X-S/T) in the HA protein of both Iranian viruses identified five sites at positions 21, 94, 128,289 and 296 in HA1, and two sites, at positions 154 and 213 in HA2, indicating a loss of one site at position 210 in comparison to Qa/HK/G1/97 prototype of the G1 sub-lineage. Furthermore, an additional potential glycosylation site was observed at residue 213 in the HA2 protein. It is known that the extent of glycosylation of HA can affect host specificity, virulence and infectivity of influenza strains and might correlate with immune pressure in the host [3].

The NA stalk length varies considerably, even within a given subtype, and correlates with the efficiency of virus replication and pathogenesis. Previous studies have shown that a longer stalk results in better replication of the virus [3]. Analysis of stalk length revealed that the isolates studied here did not show any deletions in comparison to Qa/HK/G1/97 and Dk/HK/Y280/97, prototypes of the G1 and Y280 sub-lineages, respectively. Deletions in the stalk region of NA may be required during the process of adaptation of influenza virus from wild aquatic birds to domestic poultry, particularly chickens [26].

Based on sequence analysis of binding-pocket residues in NA for neuraminidase inhibitor drugs, all of these sites were conserved and similar in Iranian isolates, and no substitution mutations were observed, therefore, the activity of NA inhibitors should remain unaffected.

The neuraminidase of IR-10 has an L403R mutation in its HB site, similar to those of isolates from Pakistan in 2005-2008 and Hong Kong in 1997, a characteristic resembling those of human H2N2 and H3N2 viruses. Mutations in the HB site of H9N2 viruses result from specific selective pressure to change, suggesting increased virus fitness in terrestrial poultry [25]. Furthermore, NA hemadsorption activity is conserved in avian viruses, and it may play a role in the maintenance of the virus in this host [21].

Seven conserved potential glycosylation sites were identified in the NA genes of both isolates. In addition, IR-98 added one glycosylation site at location 70; however, IR-10 lost a glycosylation site at this position and gained a new glycosylation site at position 44. It is known that glycosylation of NA can also be involved in the pathogenicity and virulence of influenza virus, whereas the mechanism by which an additional glycosylation site in NA may affect pathogenicity needs to be evaluated [17].

Numerous mutations within the PB2 segment have been shown to play a key role in host range and virulence. A glutamic acid at PB2 position 627 is commonly seen in AIV, while almost all human influenza A viruses possess lysine at this position. A PB2 E627K mutation can cause enhanced pathogenicity and adaptation to mammalian hosts [35]. Analysis of deduced amino acids in the PB2 protein of the studied isolates revealed that neither isolate contains an E627K mutation. The putative PB2 protein of the examined Iranian isolates displayed aspartic acid at position 701. Furthermore, a PB2 D701N substitution was also found to be responsible for replication and transmission of H5N1 AIV in mammalian hosts [12].

Major determinants of host-associated genetic signatures are located in the RNP complex genes [7, 27, 34]. In the present study, we observed three amino acid substitutions in the PA and NP genes of the examined isolates. There were two amino acid substitutions located in the PA (L356R) and NP (D372E) genes of IR-10 and one in the NP (L136M) gene of IR-98, which were changed to mammalian amino acid signatures; however, the effects of these mutations in adaptation and enhanced virulence of these isolates are not yet known.

Adamantane derivatives (amantadine and rimantadine) block the ion channel formed by the M2 protein and inhibit an early step of virus replication. The amino acid substitutions L26F, V27A, A30T, S31N and G34E in the M2 protein are known to confer resistance to the adamantanes [5, 14]. The M2 protein of IR-10 contained A27, suggesting that this virus would have resistance to amantadine. Whether this mutation was caused by natural point mutations or the use of amantadine is unknown.

Large-scale sequence analysis of AIVs revealed that the C-terminus of NS1 contains the PL motif as a potential virulence determinant. Analysis of the four C-terminal amino acids of sequences from avian, human, swine and equine isolates showed that each of them has a typical motif. The most common avian and human PL motifs were ESEV and RSKV, respectively [29]. The IR-98 isolate possessed the typical avian PL motif, whereas IR-10 contained a unique PL motif, KSEI, which is similar to what has been found recently in the Pakistani isolates Ck/PK/UDL-02/06 and Ck/PK/UDL-01/08. In a previous study, analysis of 1196 NS1 sequences revealed that only 48 avian sequences displayed isoleucine at position -1, and one human sequence carried lysine at position -4 of the PL motif [29]. The 1918 H1N1 and H5N1 viruses contain a PL motif, KSEV and EPEV, respectively, which increases virulence when introduced into a mouse-adapted influenza virus strain [19]. However, the role of the NS1 PL motif in the pathogenesis of H9N2 viruses in the poultry host remains unknown.

To our knowledge, this is the first report of complete genome sequencing of H9N2 AIV in Iran. Our results indicate that the surface glycoproteins of both isolates belonged to the G1 sub-lineage, in contrast to some of the internal genes, which were from an unknown source. The hemagglutinin cleavage site motifs of IR-98 and IR-10

isolates were RSSR and KSSR, respectively, which is characteristic of low-pathogenic AIVs. Despite the low virulence of the recent isolate, this strain carried a substitution Q226L in comparison to the early isolate, indicating its potential to infect humans. Genetic analysis also showed that IR-10 contained the mutation V27A within the M2 protein, leading to amantadine resistance. Since recombination can change the virulence or antigenicity of viruses, the close similarity of internal genes (M and NS genes of IR-10) may have a significant effect on the epidemiology and pathogenicity of the virus. Further studies are needed to obtain more information on the virulence and antigenic change of the new strain in avian and mammalian hosts.

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