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Molecular characterization of low pathogenic avian influenza H9N2 virus during co-circulation with newly-emerged highly pathogenic avian influenza H5N8 virus in Iran

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Summary. – Identification of molecular characteristics of low pathogenic avian influenza (LPAI) H9N2 virus provides insights into the evolution of this subtype due to the modulation of genomic characteristics in co-circulation with another subtype. The present study aimed to analyze the molecular and phylogenetic characteristics of the current LPAI H9N2 virus in characteristics of internal proteins are crucial for the adaptations of AIVs viruses to a new host. Since H9N2 is indigenous among poultry, continuous monitoring of viral genetic changes is needed for risk assessment of potential transmissibility to human population and emergence of new reassortant virus. domestic poultry during the emergence of new highly pathogenic avian influenza (HPAI) H5N8 virus in Iran. To this end, deep sequencing of LPAI H9N2 virus was performed on Illumina MiSeq sequencing platform and the complete sequences of avian influenza viruses were obtained from GISAID EpiFlu database. Phylogenetic analysis of the surface and internal gene segments showed that the H9N2 2018 virus was closely related to Pakistani H9N2 isolates. HA cleavage site motif sequence of the Iranian isolate was ^{317}KSSR GLF^{323}. The A/chicken/Iran/1/2018 H9N2 strain carried the amino acid substitution (Q216L), which is a mutation correlated with a shift in the affinity of the HA from avian type sialic receptors to human type. Besides surface glycoproteins, molecular

Keywords: A/H9N2; molecular characterization; A/H5N8; co-circulation; A/H5N1; Illumina MiSeq

Introduction

The prevention and control of influenza virus transmission and infection is considered as a main public health challenge. Since many migratory birds travel across the border, control of the virus movement from one country to another is difficult (Bailey *et al*., 2018). Trading of live birds, live stocks and their products further increases the virus transmission risk over various geographical regions and countries. In addition, movement of people from to countries endemic for avian influenza complicates the prevention and control of the influenza virus infections (Khan *et al*., 2007). Besides seasonal influenza, viruses not circulating in the human population but regarded as pandemic threat should also be monitored. Highly pathogenic avian influenza (HPAI) viruses like H5N1, H7N7, and H5N6 are endemic among birds in some countries, including China, with different genetic variants that exhibit antigenic variants (Brammer *et al*., 2009; Gu *et al*., 2013). Although, human infection with low pathogenic avian influenza (LPAI) viruses, namely H9N2, H7N7 and H7N9,

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Abbreviations: HA = hemagglutinin; HPAI = highly pathogenic avian influenza; LPAI = low pathogenic avian influenza; NA = neuraminidase; NGS = next generation sequencing

were reported, no human to human transmission has been observed. The recent outbreak of HPAI H5N8 virus in Europe, North America, Asia, India and Iran with no human infection report has highlighted the importance of risk assessment of LPAI H9N2 virus as an endemic virus in domestic poultry in Asia (Ghafouri *et al*., 2017; Ke *et al*., 2017; Ghabeshi *et al*., 2020).

Identification of the genomic characteristics of LPAI H9N2 virus using a comprehensive survey contributes to the understanding of the evolution of this virus subtype based on the modulation of the genomic characteristics in co-circulation with other subtypes (Hagag *et al*., 2019). In the recent decades, remarkable advances in molecular technologies, also known as next generation sequencing (NGS), have offered a powerful tool for investigating the heterogeneity of influenza genome. In addition to molecular characterization, NGS enables the detection of re-assortment events and antiviral resistance mutations anywhere in the genome. The co-circulation of the zoonotic subtypes increases the risk of generation of reassortants with unstable phenotypic properties probably dangerous for human population (Goldstein *et al*., 2018; Shi *et al*., 2019). Despite the co-circulation of HPAI (H5N1 and H5N8) and LPAI H9N2 viruses in recent decade, no natural reassortant has been detected so far among the subtypes of domestic poultry population in Iran. However, the risk of natural reassortant increased after the introduction of HPAI H5N8 virus in 2016. This study analyses the molecular and phylogenetic characteristics of the current LPAI H9N2 virus in domestic poultry during the emergence of novel HPAI H5N8 virus in Iran.

Materials and Methods

Virus detection and isolation. Chicken clinical lung tissue sample, which was confirmed to be H9N2-infected, was received from Iran Veterinary Organization in 2018. Transported in viral transport medium (VTM), the sample was homogenized and centrifuged at 1,800 x *g* for 15 min for clarity. Before the egg inoculation, the supernatants were treated with an equal volume of antibiotic cocktail (100 U/ml penicillin and 100 U/ml streptomycin) for a minimum of 1 h at room temperature. Specific-pathogen-free chicken embryonated eggs (9–11-day-old) (Razi Vaccine and Serum Research Institute, Karaj) were inoculated by chorioallantoic sac (CAS) route to isolate and

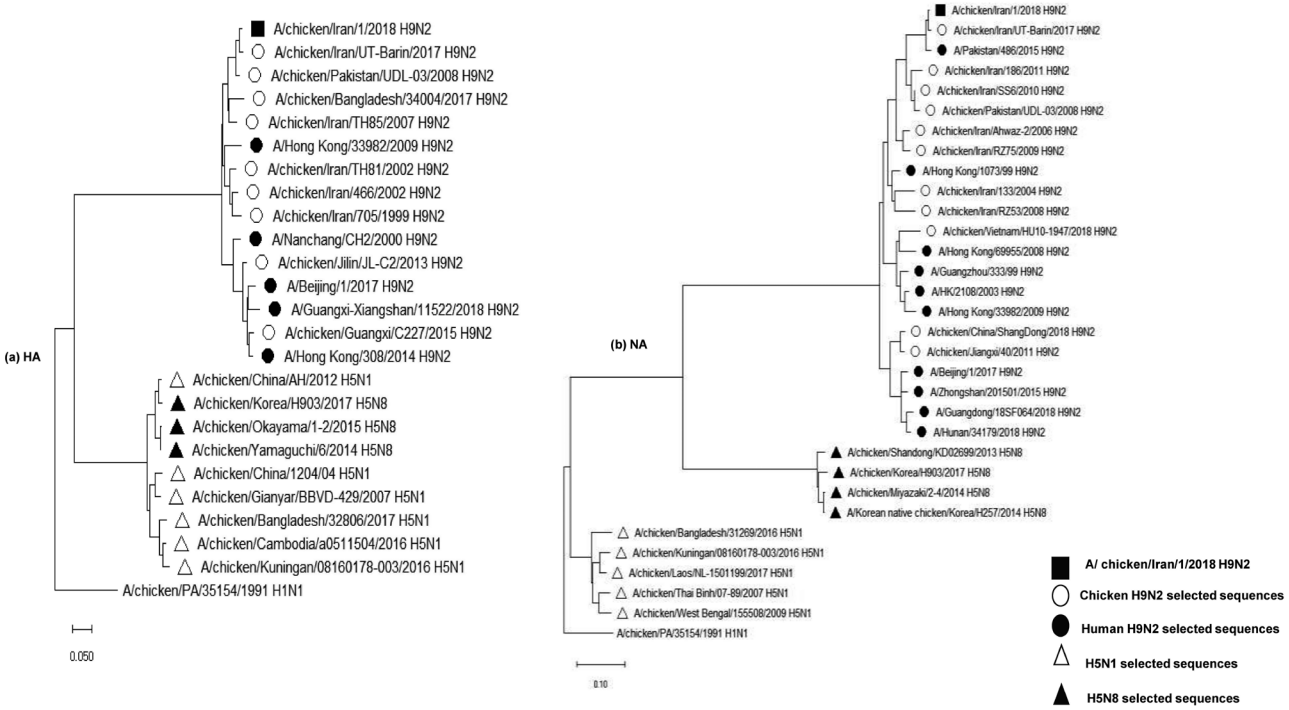

Fig. 1

Phylogenetic characterization of surface genes

Phylogenetic tree for HA **(a)** and NA **(b)** genes drawn by neighbor-joining method based on the Poisson model using MEGA X software. The selected sequences are indicated as follows: solid square: A/chicken/Iran/1/2018, hollow circle: Chicken H9N2 selected sequences, solid circle: Human H9N2 selected sequences, hollow triangle: H5N1 selected sequences and solid triangle: H5N8 selected sequences. Bootstrap values based on 1,000 replicates are shown at each main branch.

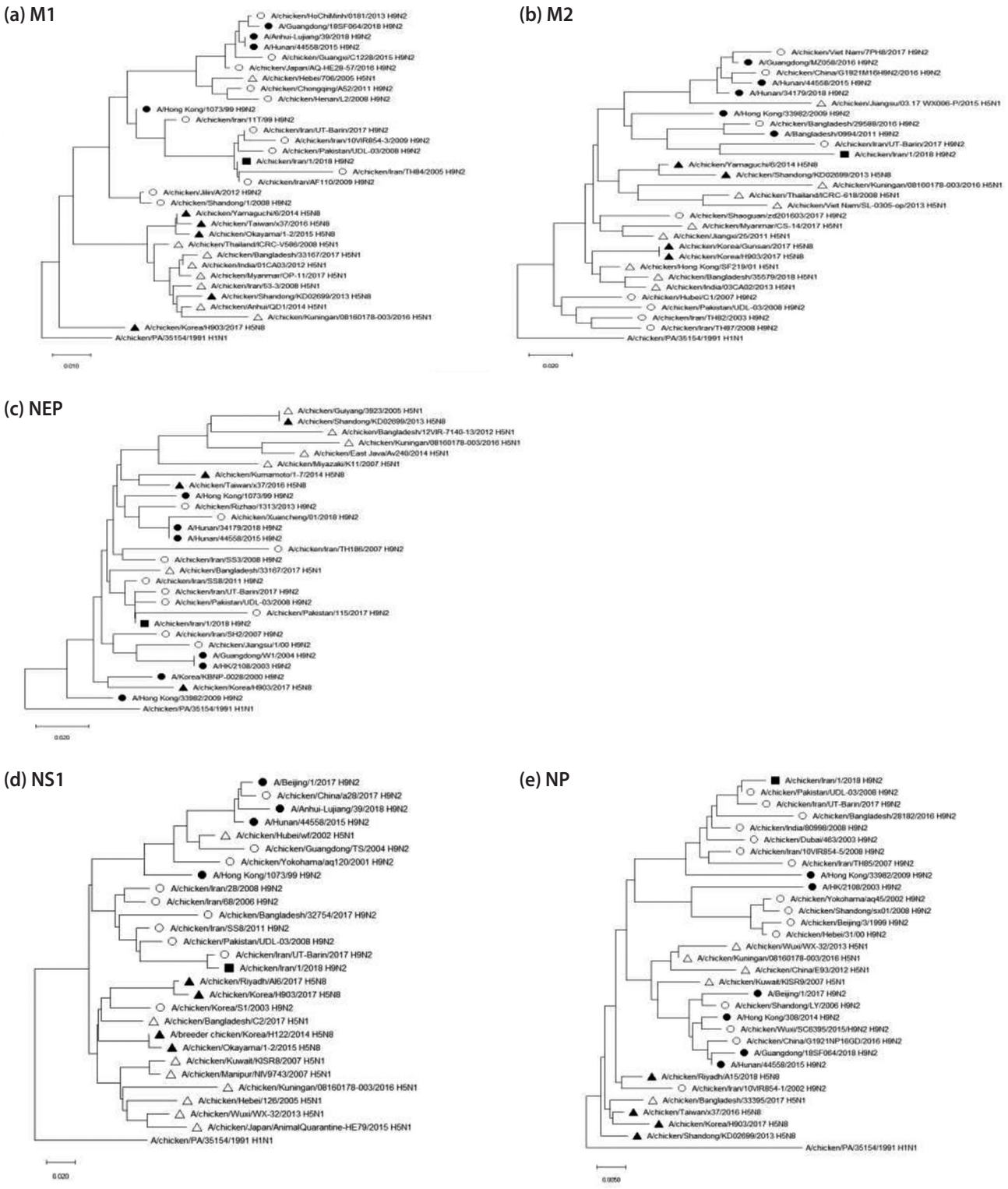

Fig. 2

Phylogenetic characterization of internal genes

Phylogenetic tree for M1 **(a)**, M2 **(b)**, NEP **(c)**, NS1 **(d)**, NP **(e)** genes drawn by neighbor-joining method based on the Poisson model using MEGA X software. The selected sequences are indicated as follows: solid square: A/chicken/Iran/1/2018, hollow circle: Chicken H9N2 selected sequences, solid circle: Human H9N2 selected sequences, hollow triangle: H5N1 selected sequences and solid triangle: H5N8 selected sequences. Bootstrap values based on 1,000 replicates are shown at each main branch.

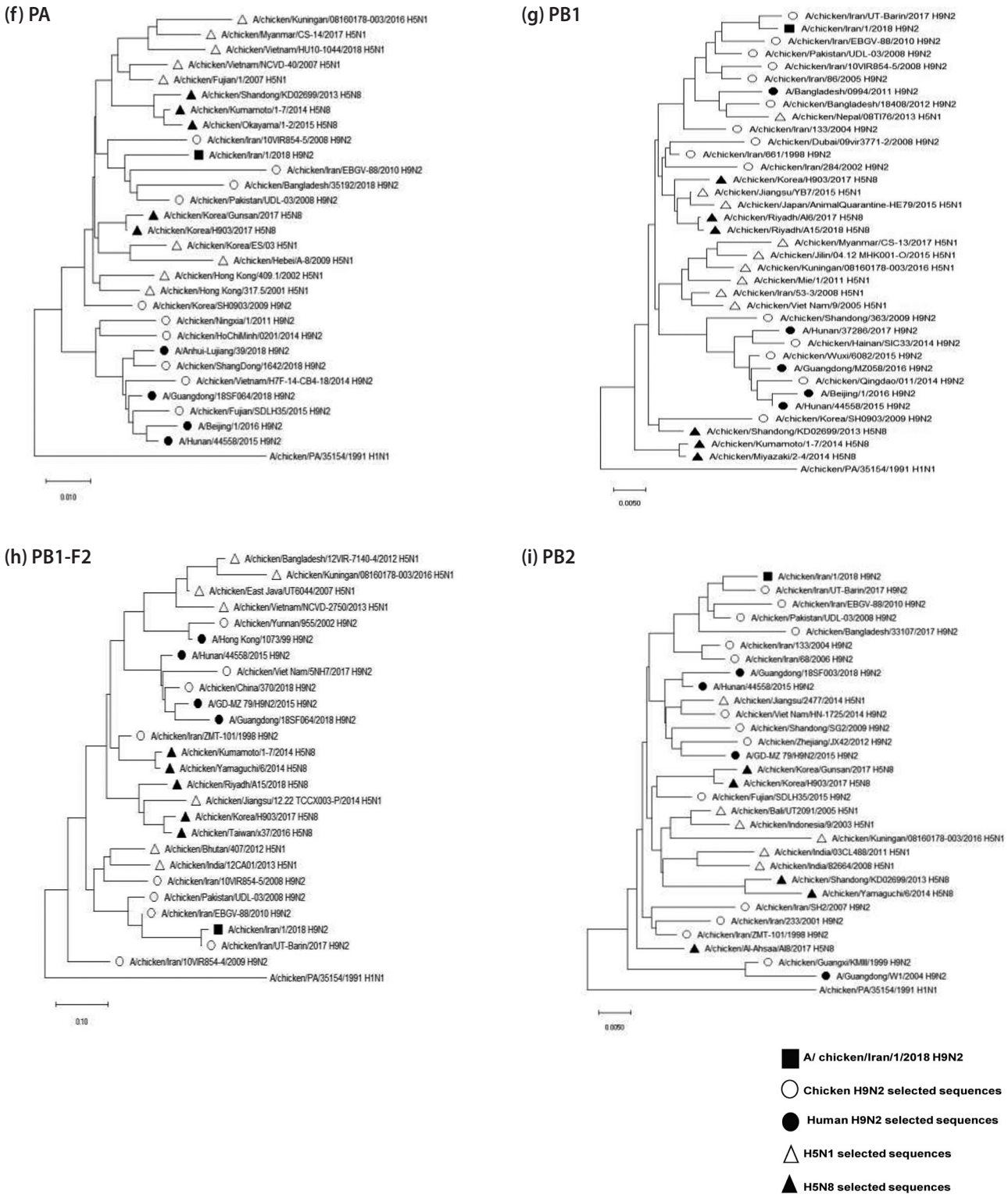

Fig. 2 (continued)

Phylogenetic characterization of internal genes

Phylogenetic tree for PA **(f)**, PB1 **(g)**, PB1-F2 **(h)** and PB2 **(i)** genes drawn by neighbor-joining method based on the Poisson model using MEGA X software. The selected sequences are indicated as follows: solid square: A/chicken/Iran/1/2018, hollow circle: Chicken H9N2 selected sequences, solid circle: Human H9N2 selected sequences, hollow triangle: H5N1 selected sequences and solid triangle: H5N8 selected sequences. Bootstrap values based on 1,000 replicates are shown at each main branch.

replicate the virus according to the World Health Organization (WHO) guidelines.

Hemagglutination (HA) assay. The infected allantoic fluids were harvested from inoculated eggs and subjected to hemagglutin activity assay based on the WHO guidelines (WHO, 2002). Freshly prepared 0.5% guinea pig red blood cells suspended in phosphate buffered saline were used for HA assay. Then, infected allantoic fluids were ultra-centrifuged for virus concentration.

RNA extraction and RT-PCR. The QIAGEN viral RNA Mini Kit (Germany) was used for RNA extraction from virus purified as mentioned in the previous section. RT-PCR was implemented with a QIAGEN One-Step RT PCR Kit (Germany) using primers for M and HA genes provided by WHO Influenza Centre, National Institute for Medical Research, London. One-Step RT PCR was done with cDNA synthesis (60°C for 1 min, 50°C for 30 min and 95°C for 15 min), DNA amplification (94°C for 30 s, 50°C for 30 s and 72°C for 1 min) for 40 cycles, and final extension (72°C for 10 min). HA subtypes were determined when the M and HA genes were positive at the same time according to the WHO guidelines (http://www.who.int/influenza/gisrs_laboratory/collaborating_centres/list/en/index.html).

Next generation sequencing. Full genome sequencing of H9N2 was carried out in the Francis Crick Institute, London, United Kingdom. To extract LPAI H9N2 virus from allantoic fluid for sequencing, the QIAamp Viral RNA Mini Kit (QIAGEN) was employed. The SuperScript III One-Step RT PCR Kit (Life Technologies) and the MBTUni12/13 universal primer set were used to indicate Multi segment reverse transcription PCR as described (Zhou *et al*., 2009). Sequencing data were generated for Iran LPAI H9N2 virus. To conduct next generation sequencing, pre-amplified reverse transcription PCR products were applied to generate the paired-end sequencing libraries by the Nextera XT DNA Sample Preparation Kit (Illumina, USA) with 96 dual index barcodes according to the manufacturer's instructions. Nextera XT transposome was applied to fragment the 100 ng input DNA, which was tagged with sequencing adapters. Library amplification was performed in a 50 µl reaction mixture under the following conditions: 98°C for 1 min, followed by 6–12 cycles of 98°C for 20 s, 60°C for 30 s, 72°C for 30 s and a final extension at 72°C for 1 min. Pooled libraries were sequenced on a 300 paired-end run using Miseq bench top next generation sequencer (Illumina, USA). Cluster density was checked by Sequencing Analysis Viewer (SAV) and optimal clustering was selected for analysis. In total, 294,078 sequences in pairs (59,446,298 nucleotides) were obtained with optimal clusters passing quality control (QC) filters (Supplementary file 1). Trimming of low-quality reads was performed using CLC Genomics Workbench (QIAGENE). The generated sequencing reads were used to generate virus genome by Templated assembly in SeqManNGen and SeqMan Pro (DNASTAR, https://www.dnastar.com/). Nucleotide sequences obtained in this study were submitted to GISAID database under accession numbers of polymerase basic 2 (PB2) (EPI1648261), PB1 (EPI1648262), polymerase acidic (PA) (EPI1648263), hemagglutinin (HA) (EPI1648264), nucleocapsid (NP) (EPI1648265), neuraminidase (NA) (EPI1648266), matrix (M) (EPI1648267), and nonstructural (NS) (EPI1648268).

Genetic and phylogenetic characterization. The assembled sequences were edited using the CLC Genomics Workbench software, version 12 (https://www.qiagenbioinformatics.com/). A search by using Basic Local Alignment Search Tool (BLAST) was performed using the Global Initiative on Sharing All Influenza Data (GISAID) platform, and sequences employed in this study were retrieved from the GISAID database for representative H5N8, H5N1, and H9N2 viruses. Alignment and identity matrix analyses were carried out using Multiple Alignment by MUltiple Sequence Comparison by Log-Expectation (MUSCLE). Phylogenetic analyses were performed using a neighbor-joining method based on the Poisson model using MEGA X software (Kumar *et al*., 2018). Trees were finally reviewed and edited using FigTree v1.4.2 software.

Results

Phylogenetic characterization of surface genes

The phylogenetic analysis of surface glycoproteins of three selected subtypes (chicken and human H9N2 isolates, H5N1 and H5N8) showed that H9N2 was placed into a cluster other than HPAI (Fig. 1). It should be noted that most of the selected sequences were from Asia. Phylogenetic analysis of the HA gene segment demonstrated that the H9N2 2018 virus was closely related to Pakistani H9N2 isolate (Fig. 1a). The NA gene segment of H9N2 2018 virus was phylogenetically grouped with the Pakistani isolates (Fig. 1b).

Phylogenetic characterization of internal genes

The remaining six genes (PB2, PB1, PA, NP, M, and NS) of the H9N2 2018 virus were closely associated with Pakistani isolate and the three selected subtypes showed a close phylogenetic relatedness (Fig. 2a-i).

Molecular characteristics of surface genes

To analyze the receptor binding site, cleavage site and glycosylation site of the Iranian isolate, the HA amino acid sequences were compared with selected human and chicken H9N2 isolates from GISAID database (Fig. 3). Most of the H9N2 isolates were >90% similar with previous isolates from Iran.

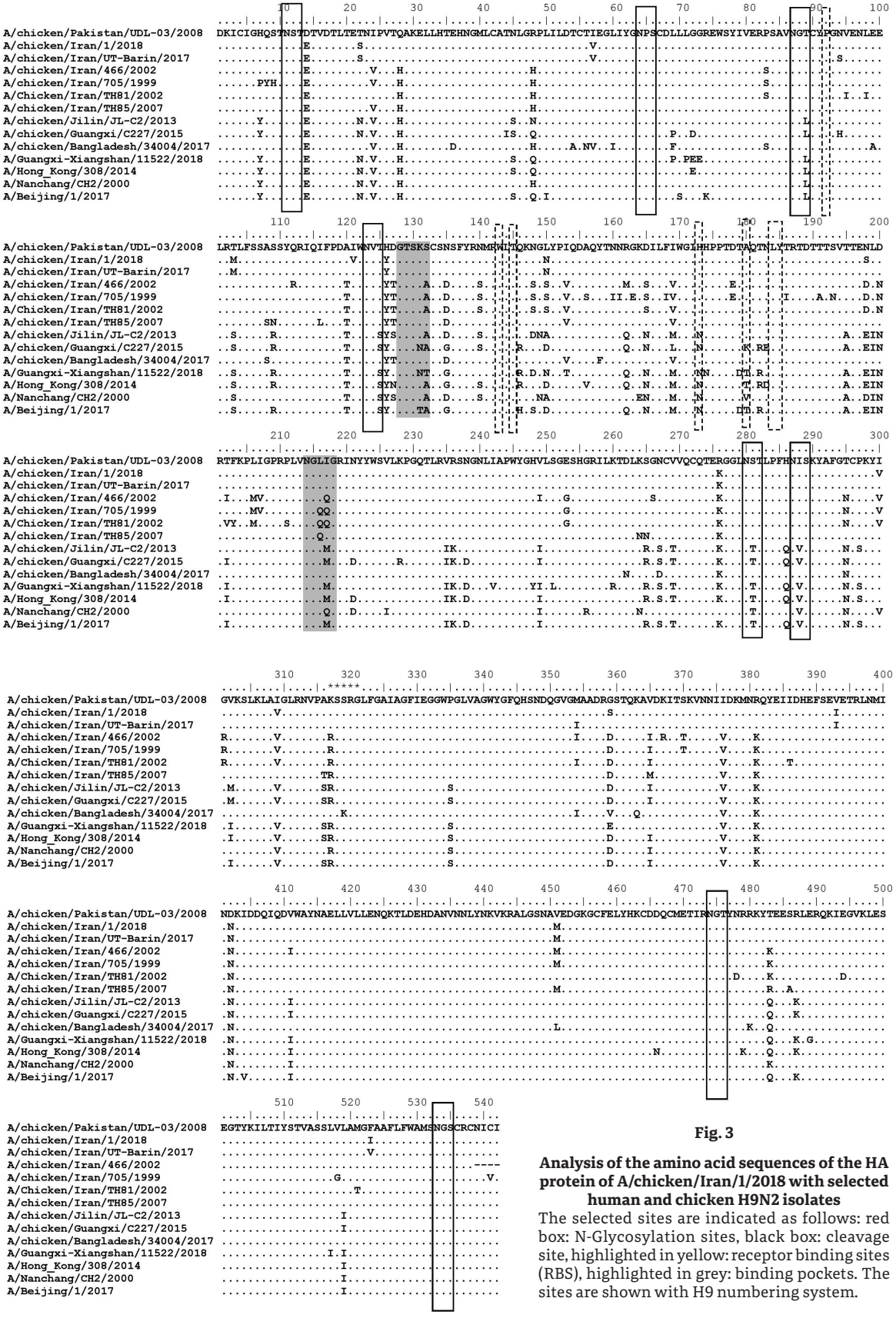

Fig. 3

Analysis of the amino acid sequences of the HA protein of A/chicken/Iran/1/2018 with selected human and chicken H9N2 isolates

The selected sites are indicated as follows: red box: N-Glycosylation sites, black box: cleavage site, highlighted in yellow: receptor binding sites (RBS), highlighted in grey: binding pockets. The sites are shown with H9 numbering system.

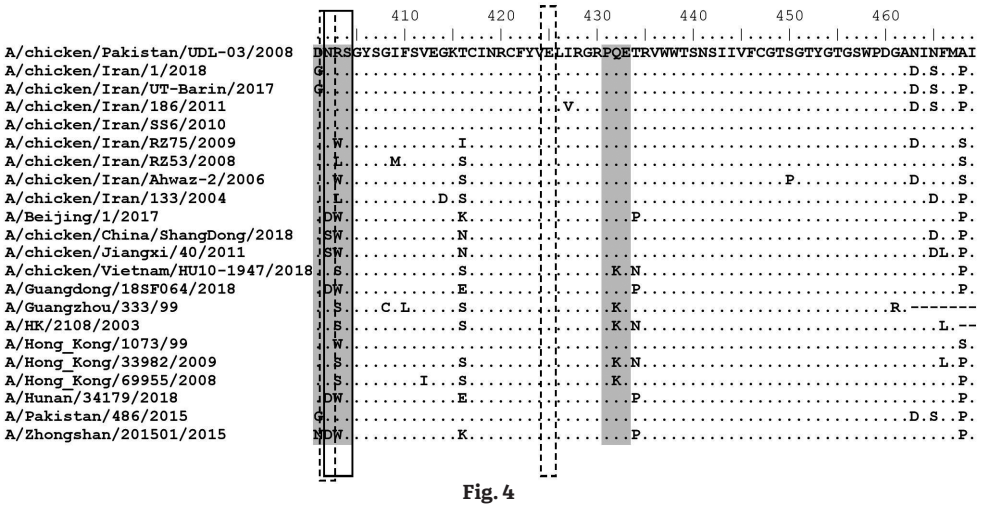

Fig. 4

Analysis of the amino acid sequences of the NA protein of A/chicken/Iran/1/2018 with selected human and chicken H9N2 isolates
The selected sites are indicated as follows: red box: N-Glycosylation sites, grey region: hemadsorption site (HB), highlighted in yellow: framework site. Position 402 was located in three sites and positions 371 and 372 were located in the HB and framework sites.

Hemagglutinin

The pathogenicity and virulence of an influenza virus are related to the HA1/HA2 connecting peptide sequence of the HA protein, specific amino acids (aa) residues at the receptor binding site (RBS), and the presence or absence of glycosylation sites around the receptor binding site.

HA cleavage site motif sequence of the Iranian isolate was ^{317}KSSR GLF^{323}, which was similar to A/chicken/Iran/UT-Barin/2017 H9N2 virus. However, most of the previous Iranian sequences, like H9N2 sequences isolated from human, had R instead of K in cleavage motif.

Major components of the receptor binding site of the HA molecule were residues at positions 92, 143, 145, 173, 180, 184, 185, 216, 217 and 218. Chicken and human H9N2 viruses showed conservation of residues P92, W143, T145, L184, Y185 in the receptor binding pocket.

The left and right edges of the binding pocket motif of the current Iranian isolates were NGLIGR and GTSKS, respectively. The A/chicken/Iran/1/2018 H9N2 strain carried the amino acid substitution (Q216L), a mutation that was correlated with a shift in affinity of the HA from avian type sialic receptors to human type and from a preference for α2, 3 link to α 2, 6 link between the sialic acid residues and galactose, whereas most previous sequences of Iran had Glu (Q) at this position. Analysis of HA protein sequences showed that selected H9N2 isolates had many potential glycosylation sites with the N-X-T/S motif (X can be any amino acid except proline), which included 11–13 (NST), 64–66 (NPS), 87–89 (NGT), 123–125 (NVT), 280–282 (NST), 287–289(NIS), 474–476 (NGT), 533–535 (NGS) (Fig. 3).

Neuraminidase

NA amino acid sequences in hemadsorption site (HB), active site, framework site and NA glycosylation sites were analyzed and compared between human and chicken H9N2 isolates (Fig. 4). Amino acids of HB site in the NA stalk include the first region (366–373), second region (399–404) and third region (431–433). Furthermore, active site including catalytic sites (R118, D151, R152, R224, E276, R292, R371, and Y406) (in N2 numbering) were conserved in all NA subtypes. On the other hand, the framework site contained aa at positions 371, 372, 402, and 425. NA glycosylation sites contain the N–X–T/S motif (in which X may be any amino acid except proline). Seven of these sites were located in the NA protein positions (44, 61, 69/70, 86, 146, 200, and 234). In A/chicken/Iran/1/2018 H9N2 strain, amino acids 367-370-372 on loops were substituted by EST. G and Y substitutions were also found at residues 401, 406, respectively. The NA active site contained conserved sequences with no mutations. However, the framework site contained 371, 372, 402, and 425 positions with A372T mutation. The NA genes of the Iranian isolate contained eight glycosylation sites, at positions NSS44, NIT61, NGT69, NWL86, NGT146, NAT200, NGT234 and 402NRS. Position 402 was located in three sites, and positions 371 and 372 were located in the HB and framework sites (Fig. 4).

Molecular characteristics of internal proteins

HPAI and LPAI viruses are very similar in their six internal genes. Therefore, internal gene analysis was performed for H5 and H9 subtypes and the results are

Table 1. Analysis of the amino acid sequences of internal proteins of selected H5 and H9 subtypes (PB2, PB1 and PA (a), NP, NS1 and NEP (b), M1 and M2 (c))

(a)

Selected HPAI and LPAI viruses	PB2								PB1		PA		
	526	543	627	655	661	701	702	714	384	386	327	631	669
A/chicken/Pakistan/UDL-03/2008_H9N2	K	E	E	V	A	D	K	S	A	R	K	S	V
A/chicken/Iran/1/2018_H9N2	K	E	E	V	A	D	K	S	T	R	E	S	V
A/Hunan/44558/2015_H9N2	K	E	E	V	A	D	K	S	S	R	V	G	V
A/chicken/Kuningan/08160178-003/2016_H5N1	R	E	E	V	A	D	K	S	L	K	E	S	V
A/chicken/Korea/H903/2017_H5N8	K	E	E	V	A	D	K	S	S	R	E	G	V

(b)

Selected HPAI and LPAI viruses	NP					NS1					NEP
	422	430	442	455	480	38	41	92	125	PDZ binding motif 227-230	16
A/chicken/Pakistan/UDL-03/2008_H9N2	R	K	T	D	D	R	K	D	D	KSEI	M
A/chicken/Iran/1/2018_H9N2	R	K	T	D	D	R	K	D	D	KPEI	M
A/Hunan/44558/2015_H9N2	R	T	T	D	D	R	K	D	D	EPEV	M
A/chicken/Kuningan/08160178-003/2016_H5N1	R	T	T	D	D	R	K	D	D	ESEV	M
A/chicken/Korea/H903/2017_H5N8	R	T	T	D	D	R	K	D	D	GSEV	M

(c)

Selected HPAI and LPAI viruses	M1					M2								
	15	115	121	137	240	17	25	26	27	30	31	34	38	50
A/chicken/Pakistan/UDL-03/2008_H9N2	I	V	T	T	Y	C	P	L	V	A	S	G	L	C
A/chicken/Iran/1/2018_H9N2	I	V	T	T	Y	Y	H	L	V	A	N	G	L	Y
A/Hunan/44558/2015_H9N2	I	V	T	T	Y	C	P	L	V	A	N	G	L	C
A/chicken/Kuningan/08160178-003/2016_H5N1	I	V	T	T	Y	C	P	L	A	A	N	G	L	F
A/chicken/Korea/H903/2017_H5N8	V	V	T	T	Y	C	P	L	V	A	S	G	L	C

presented in Tables 1a-c. Amino acids at positions 526, 543, 627, 655, 661, 701, 702 and 714 of PB2 could serve as a marker of the virulence phenotype and host range. Analysis of selected sequences in the above-mentioned positions showed the similarity of the selected HPAI and LPAI viruses (Table 1a). The most important positions in PB1 and PA polymerase complex are shown in Table 1a. The nucleoprotein (NP) of selected HPAI and LPAI viruses retained the conserved residues of R422, K430, T442, D455 and D480 (Table 1b).

As the analysis of the non-structural (NS1) protein showed, the five amino acid (80TIASV84) deletions were not observed in the isolate studied here. The presence of this motif resulted in greater length of NS1 protein and inclusion of the "KPEI" PDZ ligand (PL) C-terminal motif. In addition, no D92E mutation required for high virulence was observed in this motif. The RNA-binding domain further indicated conserved amino acid residues of P31, D34, R35, R38, K41, G45, R46 and T49. However, the effector domain carried the R184G substitution. M16I mutation in NEP, which plays a critical role in crossing the species barrier, was not observed in the selected sequences.

Important positions of Matrix protein (M1) in replication, entry, assembly and budding, which were similar in all selected sequences, are demonstrated in Table 1c. Despite the similarity in most of the drug-resistance positions of M2 selected sequences, the H9N2 Iranian isolate showed C17Y, P25H and C/F50Y (Table 1c).

Discussion

Deep sequencing analysis was used for characterization of genomic diversity of field sample infected with the A/H9N2 virus. Because of current co-circulation of HPAI (H5N1, H5N8) and LPAI (H9N2) viruses in Iran, ac-

tive surveillance of enzootic avian influenza A H9N2 is required. Although no new reassortant has been detected by phylogenetic relatedness and nucleotide identity in this study, the circulation of new reassortant H9N2 viruses cannot be neglected. Occurrence of the natural reassortment between avian influenza viruses of H5N8 and H9N2 and the emergence of HPAI (H5N2) virus in Egypt demonstrate the increasing risk of of natural reassortants emerging (Hagag *et al*., 2019). According to the previously published studies (Sealy *et al*., 2018; Ghabeshi *et al*., 2020), all genome segments of the virus might have key role in host adaptation and pathogenicity of A/H9N2. Several elements of the surface glycoproteins hemagglutinin (HA) and neuraminidase (NA) are involved in the virulence and pathogenicity of A/H9N2. Whereas most previous sequences from Iran have Glu (Q) at position 216, the A/chicken/Iran/1/2018 H9N2 strain carried the substitution (Q216L). This mutation is correlated with a shift in the affinity of the HA from avian to human type and from a preference for α 2, 3 to α 2, 6 link between the sialic acid residues and galactose.

The cleavage site is an important component in understanding pathogenicity of A/H9N2 in co-circulation with other subtypes. The highly pathogenic H5 and H7 AIVs have the RXR/KR motif (X = a non-basic residue), while RSRSSR motifs are important in LPAIVs. Recent A/H9N2 viruses can gain dibasic and tribasic motifs resembling HPAIV in the HA cleavage site. The polybasic cleavage site in highly pathogenic AIVs enables the cleavage of HA by a family of ubiquitous subtilisin-like proteases. However, the monobasic cleavage site in LPAIVs limits HA cleavage by trypsin-like enzymes, leading to localized and mild infection in the respiratory and intestinal tracts of birds. In the present study, amino acid sequence of the HA cleavage site of the current A/H9N2 is "KSSR", which differs from the RSSR motifs of candidate vaccine viruses for A/H9N2 (Tian *et al*., 2012; Jegede *et al*., 2018).

The second surface glycoprotein (NA) has an enzymatic role in removing sialic acid from the host cell and viral glycoproteins, allowing the release of progeny virus particles at the final stage of infection (McAuley *et al*., 2019). Among all subtypes and types of influenza viruses, the NA active site is conserved and is considered as an appropriate choice for antiviral therapy. Moreover, since NA plays various roles in host range, monitoring pathogenicity and antiviral properties of mutations in the NA gene is required as they may result in a public health threat (Lv *et al*., 2015; McAuley *et al*., 2019). NA of the current Iranian isolates (A/chicken/Iran/UT-Barin/2017 H9N2 virus and A/chicken/Iran/1/2018 H9N2 virus) is similar to that of previous ones, although they carry substitutions in the HB site in E367 and G401 positions. The mechanism, by which an additional glycosylation site in NA may affect pathogenicity, is obscure but supposed to be linked to the HA cleavage in cells. For example, the highly glycosylated NA of the highly pathogenic virus may facilitate the increasing activation of the host proteases required to cleave the HA of the virus. The glycosylation site 402, which was described previously as a characteristic of H9N2 viruses, was observed in Iranian virus and in contrast, site 402 was not present in some viruses of human origin. Addition or loss of potential glycosylation may contribute to the increase in virulence caused by change in sialidase activity and antigenicity (Chen *et al*., 2012; Mosaad *et al*., 2017).

In addition to surface glycoproteins, molecular characteristics of internal proteins are crucial for the adaptation of AIVs viruses to a new host. One of the most important internal proteins found in the studies on the host range is PB2. Sequence analysis of PB2 in this study was similar to those previously reported for A/H9N2 in Iran such as E627, D701 and S714 positions (Soltanialvar *et al*., 2009). However, Alanine (A) at position 661 and Lysine (K) at position 702 were discovered to be identical to H5N1 viruses and human A/H9N2 isolates. According to Arai *et al*., E627V, E543D, A655V and K526R mutations confirm the effect of PB2 on changing host range from birds to mammals. A655V mutation was further detected in A/H9N2 isolates in the present study. The high prevalence of PB2 mutations during the evolution of A/H9N2 indicated that this protein with a prominent role in the mammalian adaptation could be considered as a vital component in the risk assessment of AIVs (Sediri *et al*., 2016; Arai *et al*., 2019). The polymerase complex consists of three subunits PB2, PB1 and PA. Many of the mutations in PA and PB1 genes are different in human viruses and avian viruses. Some of these substitutions are assumed to play a role in host adaptation (Soltanialvar *et al*., 2009; Welkers *et al*., 2019). According to Arai *et al*. (2016), 17 mutations in PA gene and 13 mutations in the PB1 could be sufficient to help the virus cross the host species barrier. E327K, G631S and A669V have been reported in many A/H9N2 Iranian isolates in the PA C-domain to interact with PB1 N-domain. Based on the previous studies, the presence of L384T and K386R mutations in the PB1 of the recent A/H9N2 located in Cap binding domain (residues 318–483) plays an important role in the proper configuration of the polymerase complex through the interaction of PB1 with vRNA promoter (Arai *et al*., 2016). PA-X and PB1-F2 are generated by ribosomal frame shifting through the translation of PA and PB1 mRNAs, respectively. Interestingly, a novel polymorphism is found in the PB1-F2 open reading frame, which results in truncations in the protein as previously reported by Zaraket *et al*. in case of human viruses (Chakrabarti *et al*., 2013; Zaraket *et al*., 2016). This new polymorphism leads to the lack of single mutation S66N that plays a crucial role in pathogenicity

of AIVs. However, none of the recent A/H9N2 indicate that L201Stop and D108A mutations are associated with pathogenicity. Therefore, the effects of these mutations on altering the host range can be multifunctional.

Nucleoprotein (NP) sequence analysis of the current A/H9N2 showed that the positions of R422, K430, T442, D455 and D480 were conserved (Tombari *et al*., 2011). NP of Iranian viruses isolated during co-circulation of H9N2, H5N1 and the newly-emerged H5N8 and the previous Iranian H9N2 isolates were highly similar.

The NS gene undergoes alternative splicing by host splicing machinery, which produces NS1 protein from the un-spliced transcript and NEP protein from the spliced transcript. Although molecular analysis of the NS1 in this study showed no change in positions 38 and 41, a variation at position 92 of NS1 was detected as a result of substitution of glutamic acid by aspartic acid, which abolished the pathogenicity of H5N1 viruses. The D125G mutation was also associated with switching host range from avian to mammalian. The presence of the PDZ motif in NS1 protein can modulate viral replication in a strain and host-dependent manner (Selman *et al*., 2012; Abdelwhab *et al*., 2013). Based on these results, the PDZ motif has KPEI sequence, which is a combination of two LPAVIs PDZ motifs (KSEI) and human/HPAIVs PDZ motifs (EPEV). The NEP is another alternative splicing product of the NS gene that is required as a new adaptive factor for overcoming polymerase restriction and facilitating adaptation to the new host (Paterson *et al*., 2012). The single adaptive mutation M16I may reflect a possibility of crossing the species barrier from avian to mammals. A/H9N2 isolated in this study has M in position 16 and is similar to other Iranian and Asian strains.

The M2 proton channel is a target of the adamantine class of drugs. Based on the previous research, drug resistance and the presence of some mutations of M2 protein are correlated. Schnell and Chou (2008) showed that amino acid substitutions at 26, 27, 30, 31, 34 and 38 positions, which are located over helical turns of M2 transmembrane domain, created adamantine-resistant mutants. The detected S31N mutation in A/H9N2 was similar to those found by Yavarian *et al*. (2009) in A/H3N2 circulating in Iran during 2005–2008 (Schnell *et al*., 2008; Malekan *et al*., 2016). In this study, C17Y, P25H and C50Y mutations were firstly observed in A/H9N2 Iran. Therefore, monitoring variations in this surface protein is essential for awareness on drug resistance and appropriate response to emerging strains and new epidemics in risk assessment of AIVs. Matrix protein 1 (M1) is a critical protein in virus replication from virus entry to assembly and budding of influenza viruses. Positions 15, 115, 121, 137 and 240 in the M1 protein discriminate human strains from avian (Shaw *et al*., 2002; Qiang *et al*., 2018). Based on our results, Iranian A/H9N2 strains harbor V15I substitution in the membrane binding region of M1, supporting the results of the previous studies.

Although H9 strain received more attention due to its enzootic nature in poultry, the H9 subtypes can create new reassortant viruses and be of concern for human health in the current co-circulation with HPAIVs (H5N8, H5N1). Therefore, co-circulation of avian influenza viruses in poultry fields has created the concern that H9N2 viruses may evolve like some H5 and H7 viruses and create new reassortant. In the present study, LPAI Iranian H9N2 strain isolated during co-circulation of HPAI (H5N1, H5N8) viruses was characterized. Since H9N2 is indigenous among poultry, viral genetic changes should be continuously monitored for risk assessment of potential transmissibility to human population and emergence of new reassortant virus.

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