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RESEARCH ARTICLE

Ongoing genetic evolution of H9N2 avian influenza viruses in Iranian industrial poultry farms

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ABSTRACT

Despite the use of wide-scale vaccination programmes against the H9N2 virus, enzootic outbreaks of H9N2 avian influenza (AI) have often occurred and caused serious nationwide economic losses, particularly in broiler chickens. In this study, the haemagglutinin (HA) and neuraminidase (NA) genes of nine recent H9N2s and a common vaccine strain were fully sequenced and compared with other representative Iranian viruses. Phylogenetic analysis revealed that all Iranian viruses were grouped into the G1 sub-lineage with different clusters in which recent isolates (2014–2017) formed a distinct cluster compared to the vaccine group (1998–2004). All Iranian H9N2s exhibited low pathogenicity AI connecting peptide feature with an R/KSSR motif. Amino acid 226, located in the 220 loop of the receptor binding site, was leucine among the recent Iranian viruses, a characteristic of human influenza viruses. With an overall gradual increase in the genetic diversity of H9N2s, Bayesian skyline plots of Iranian HA and NA genes depicted a fluctuation and a relative stable situation, respectively. It is recommended to apply constant surveillance to assess any increase in viral human adaptation and evolutionary changes in circulating field H9N2s. Moreover, antigenic characterisation of the prevailing H9N2 viruses seems to be necessary for evaluating the possible antigenic drift from the vaccine strain.

KEYWORDS

H9N2 avian influenza virus, HA gene, NA gene, phylogenetic analysis, evolution, chicken, Iran

INTRODUCTION

Avian influenza virus (AIV) subtype H9N2 is known as one of the most dominant low-pathogenicity (LP) AIVs in the poultry industry all over the world (Song et al., 2011). This virus was first isolated from turkeys in Wisconsin in the mid-1960s (Homme and Easterday, 1970). Subsequently, in the 1990s, extensive spread of the H9N2 virus was reported from various countries of Europe, Asia, Africa and the Middle East (Song et al., 2011). Regardless of broad vaccination in those days, nowadays subtype H9N2 has become

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Table 1. Information related to H9N2 viruses used in this study

Viruses	Production type	Place of isolation	Isolation date	Accession numbers	
				HA gene	NA gene
Ck/Isfahan/FBM-1/13	Broiler	Isfahan, Iran	August 2013	MN319500	MK968543
Ck/Isfahan/FBM-2/14	Broiler	Isfahan, Iran	November 2014	MN319501	MK968544
Ck/Tehran/FBM-3/14	Broiler	Tehran, Iran	December 2014	MN319502	MK968545
Ck/Alborz/FBM-4/17	Broiler	Alborz, Iran	November 2017	MN319503	MK968546
Ck/Hamadan/RIV-1/17	Broiler	Hamadan, Iran	September 2017	MN319504	MK968547
Ck/Yazd/RIV-2/17	Broiler	Yazd, Iran	September 2017	MN319505	MK968548
Ck/Markazi/RIV-3/17	Broiler	Markazi, Iran	November 2017	MN319506	MK968549
Ck/Mazandaran/RIV-4/17	Broiler	Mazandaran, Iran	December 2017	MN319507	MK968550
Ck/Mazandaran/RIV-5/17	Broiler	Mazandaran, Iran	December 2017	MN319508	MK968551
Ck/Tehran/Marand/98	Broiler	Tehran, Iran	June 1998	MN319514	MK968557

enzootic in industrial poultry of several Asian, Middle Eastern and African countries (Alexander, 2007; SJCEIRS Working Group, 2013). Occasionally the H9N2 virus broke through the species barrier and infected humans, causing influenza-like symptoms and mild upper respiratory tract infections (Guo et al., 1999; Saito et al., 2001). Furthermore, the H9N2 virus is able to act as a donor of whole six internal genes or less for zoonotic AIVs such as the H5N1, H7N9, H10N8 and H5N6 subtypes, posing a threat to human life (Guan et al., 1999; Saito et al., 2001; Qi et al., 2014; Pu et al., 2015).

Generally, two well-defined geographical lineages of H9N2 viruses exist, the North American and the Eurasian. The Eurasian lineage is further divided into four main sub-lineages of G1 (Quail/Hong Kong/G1/97), Y280 (Duck/Hong Kong/Y280/97 and Chicken/Hong Kong/G9/97), Y439 (Chicken/Korea/38349-p96323/96 and Duck/Hong Kong/Y439/97) and F/98 (Chicken/Shanghai/F/98) (Guan et al., 1999; Matrosovich et al., 2001; Butt et al., 2010). In another classification, H9N2 viruses isolated from 1998 to 2010 in Central Asia and the Middle East were sorted into four distinct groups (A, B, C and D). From these categories, the two former groups (A and B) are widely distributed through the Asian countries (Fusaro et al., 2011).

H9N2 AIV was enzootic in Iran for two decades after the first recognition of the disease in 1998. Since that time, vaccination has been adopted as an alternative approach to mitigate the impact of H9N2 viruses in the industrial poultry of Iran (Bashashati et al., 2013). Nevertheless, large outbreaks of the disease continue to occur due to the antigenic drift of H9N2s in which antigenic variants arise (Park et al., 2011). Moreover, unlike the seasonal influenza vaccine for human usage, H9N2 vaccines have never been updated in Iran to better match circulating viruses since the first isolation of the virus. In this study, we elucidate the complete haemagglutinin (HA) and neuraminidase (NA) genes of 9 recent isolates of H9N2 viruses along with a vaccine strain (Ck/Tehran/Marand/98). To understand the evolutionary attributes, phylogenetic characterisation and molecular analysis of the studied strains were performed and compared with Iranian nucleotide sequences publicly available from GenBank.

MATERIALS AND METHODS

Virus isolation and propagation

A total of ten H9N2 viruses were obtained from the Department of Avian Disease Research and Diagnostic, the Razi Vaccine and Serum Research Institute (Table 1). The recent H9N2s were recovered from the respiratory system (trachea and lung) of broilers with severe respiratory signs and high mortality. For virus propagation, all isolates were inoculated into the allantoic cavity of 9-day-old specific-pathogen-free (SPF) embryonated chicken eggs (Venkey's, Maharashtra, India). After 48 h of incubation, the amnio-allantoic fluids were harvested and tested for the presence of H9N2 virus by haemagglutinin activity (OIE, 2019).

Extraction of viral genomic RNA and reverse transcription (RT)-PCR

The allantoic fluid from infected embryonated eggs was extracted and purified using the High Pure Viral RNA kit (Roche Life Science, Mannheim, Germany), in accordance with the manufacturer's recommendations. The purity of the extracted RNA was assessed by calculating the ratio of readings at 260 and 280 nm.

Next, cDNAs of the studied viruses were synthesised with uni12 primer (5′-AGCAAAAGCAGG-3′) using the RevertAid cDNA synthesis kit (Thermo Fisher Scientific, Massachusetts, US), following the user manual. The full length of HA and NA genes of the above-mentioned H9N2s were amplified using influenza virus universal primers described previously (Hoffmann et al., 2001). The PCR mix was performed in a 50-µl reaction mix containing 33 µl of nuclease-free water, 5 µL of 10× Ampliqon Ammonium Buffer, 1 µL of 25 mM MgCl2, 2 µL 10 mM dNTP mix, 2 µL of each primer (10 µM), 1 µL of 2.5 units/µL Ampliqon AccuPOL™ DNA Polymerase (AMPLIQON, Odense M, Denmark) and 4 µL of cDNA. The PCR thermal profile was set up at 95 °C/2 min, and 35 cycles at 95 °C/30 s, 50 °C/30 s and 72 °C/4 min followed by a final extension of 72 °C/5 min.

Cloning and transformation

After running on 1% agarose gel, each amplicon was excised and purified by the GeneJET Gel extraction Kit (Thermo Fisher Scientific, Massachusetts, US). The purified PCR fragments were ligated into pJET1.2/blunt according to CloneJET PCR Cloning Kit instructions (Thermo Fisher Scientific, Massachusetts, US) and then transformed into competent *Escherichia coli* TOP10 cells by exposing to heat shock at 42 °C for 1 min. The transformants were spread on Luria-Bertani (LB) agar supplemented with 50 µg/mL ampicillin. Analysis of recombinant clones containing the expected insert was performed by either colony PCR amplification using the same primers for full-gene amplification or restriction endonuclease BglII. The positive plasmids were extracted from an overnight culture of bacteria using the High Pure Plasmid Isolation Kit (Roche Life Science, Mannheim, Germany).

Nucleotide sequencing and phylogenetic analysis

Recombinant plasmids were submitted to a commercial sequencing service (Faza Pajooh Company, Tehran, Iran) for sequencing, using the pJET1.2 universal forward and reverse primer (HA and NA genes), as well as two internal primers for the HA gene (primer sequences will be provided upon request). All sequence data were assembled and analysed using the BioEdit software version 7.0.5 (Hall, 1999). Nucleotide and deduced amino acid sequences were aligned with the Clustal W method in the MEGA software version 7.0.26 (Kumar et al., 2016). BLAST analysis (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was performed to retrieve identical sequences related to our queries from the GenBank database. Phylogenetic analyses were carried out using the neighbour-joining method with the maximum composite likelihood model by calculating 1,000 replicates for bootstrap value in the MEGA 7.0.26 software (Kumar et al., 2016). Potential N-linked glycosylation sites in HA and NA protein were predicted using the NetNGlyc 1.0 server (http://www.cbs.dtu.dk/services/NetNGlyc).

Evolutionary analysis

To identify the natural selection on the coding sequences of HA and NA genes, the ratio of non-synonymous to synonymous substitutions ($\omega = dN/dS$) was measured using single likelihood ancestor counting (SLAC) in the web-based Datamonkey suite (Weaver et al., 2018). The dataset is composed of 59 Iranian HA and 65 NA complete coding sequences retrieved from GenBank for the period of 1998–2017. The ratio of ω for each amino acid site in the HA and NA proteins was used to scan for evidence of positive or negative selection with $\omega > 1$, $\omega = 1$ and $\omega < 1$ indicating a positive selection, neutral evolution and negative selection, respectively. The *P*-value threshold of 0.05 was set for evidence of positive and negative selection.

The dataset of Iranian HA and NA sequences was subjected to recombination analysis with recombination detection program (RDP) version beta 4.56 (Martin et al., 2015). Seven algorithms including RDP (Martin and Rybicki, 2000), GENECONV (Padidam et al., 1999), BootScan (Salminen et al., 1995), MaxChi (Smith, 1992), Chimaera (Posada and Crandall, 2001), SiScan (Gibbs et al., 2000) and 3Seq (Boni et al., 2007) were used for finding evidence of recombinant sequences. If a recombination was detected with at least three algorithms in any analysed sequence, it was regarded as a valid recombination and removed from the dataset (HA = 58 and NA = 61) used for Bayesian skyline plot reconstruction available in the BEAST software, version 1.10.4 (Suchard et al., 2018). Because of the higher performance of the codon-based SRD06 nucleotide substitution model (Fusaro et al., 2011), we employed this model with an uncorrelated lognormal relaxed clock and gamma distributed rate under a skyline coalescent tree prior. The Markov chain Monte Carlo (MCMC) for both genes was run by setting 50 million steps with sampling every 10,000 generations. To estimate polydynamic history genetic changes within the Iranian HA and NA sequences of H9N2 viruses, two Bayesian skyline plots were reconstructed using the tracer software, version 1.7.1 (Rambaut et al., 2018).

Nucleotide sequence accession numbers

The twenty sequences of H9N2 HA (MN319500–MN319508, MN319514) and NA (MK968543–MK968551, MK968557) genes obtained in this study have been deposited in GenBank.

RESULTS

Study of genetic identities and phylogenetic analysis

According to BLAST searches, the studied vaccine strain (Marand) was more similar to viruses previously isolated from the Japanese parakeet in 1997. However, Pakistani strains were the closest relative to other studied sequences. Concerning HA and NA genes of FBM-1, a higher per cent of identity was shown with Iranian H9N2 viruses isolated in 2010 and 2011, which are closely related to H9N2 strains isolated from Pakistan between 2005 and 2008 (Bashashati et al., 2013).

Pairwise comparison of nucleotide sequences of the Iranian HA and NA genes revealed that identities among viruses ranged from 88.2 to 99.8% and 93.1 to 100%, respectively (data not shown). The deduced amino acid sequences of HA and NA shared 91–99.8% and 88.6–100% homology, respectively (data not shown). At the nucleotide level, all studied viruses except FBM-1 had about 12% divergence with the current vaccine strain, which showed quite a low homology with significant genetic distances.

All studied isolates and published Iranian sequences available in GenBank were subjected to phylogenetic analysis (Fig. 1). The Iranian HA sequences were clustered into 4 distinct genetic groups in G1/like sub-lineage, which contained viruses isolated from different periods of times: (a) 1998–2004, (b) 2005–2009, (c) 2010–2013 and (d) 2014–2017 (Fig. 1a). In the NA phylogenetic tree, there were five distinct clusters that are composed of H9N2s in (i) 1998–2004, (ii) 2006–2008, (iii) 2009–2012, (iv) 2010–2013 and

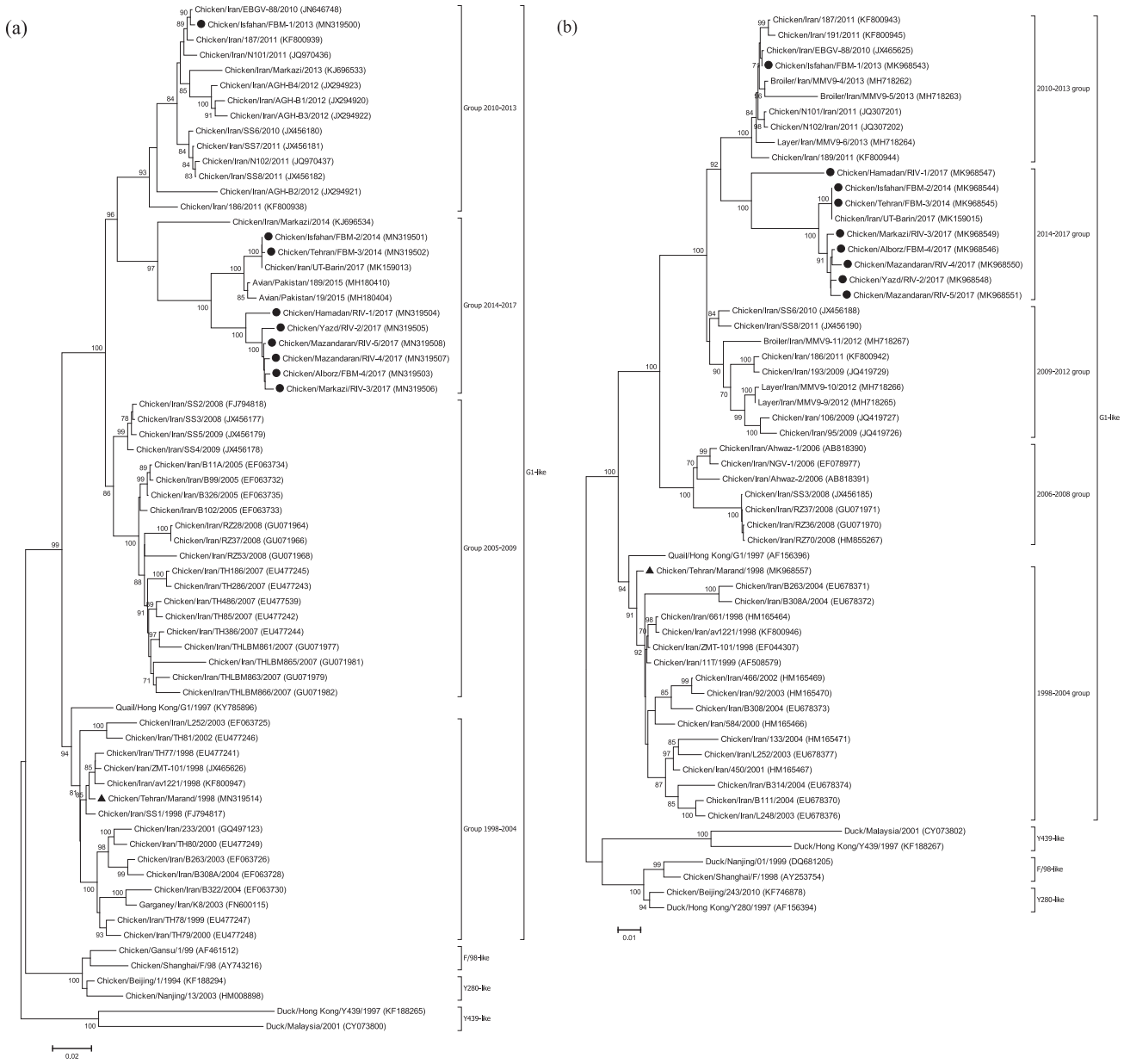

Fig. 1. Phylogenetic tree for HA (a) and NA (b) genes of the studied H9N2 viruses along with other Iranian and representative viruses retrieved from GenBank. Both trees were generated in MEGA software version 7.0.26 using the neighbour-joining method with the maximum composite likelihood model. The studied isolates are marked with black circles. The black triangle indicates the vaccine strain. Bootstrap values ≥70 are shown next to the branches

(v) 2014–2017 (Fig. 1b). As can be seen, the topology of both HA and NA phylogenetic trees is very similar.

Evolutionary features

Positive and negative selection pressures of the coding sequence of HA and NA were estimated using the Datamonkey software as described above. Purifying selection was observed in Iranian HA and NA genes with mean dN/dS values of 0.177 and 0.231, respectively. The only positively selected amino acid substitution found among both studied genes of Iranian H9N2 viruses was located at position 190 (henceforth, H3 numbering was denoted throughout the manuscript) in HA protein by a P-value threshold of <0.05. In this regard, 95 and 72 amino acid residues in the HA and NA genes of Iranian H9N2s were found to be under negative selection, respectively.

The genetic diversity of Iranian HA and NA genes showed a slight increase over a period of 20 years (Fig. 2). After the first record of the H9N2 AIV in 1998, the viral population size remained relatively stable until 2001. Fluctuations in viral diversity were observed in HA Bayesian skyline plot as three phases. Regarding Iranian HA genes, effective population size had experienced a period of instability since 2005 that lasted for about a decade (Fig 2a). Bayesian skyline plot analysis for the NA genes of the Iranian H9N2 viruses did not show any

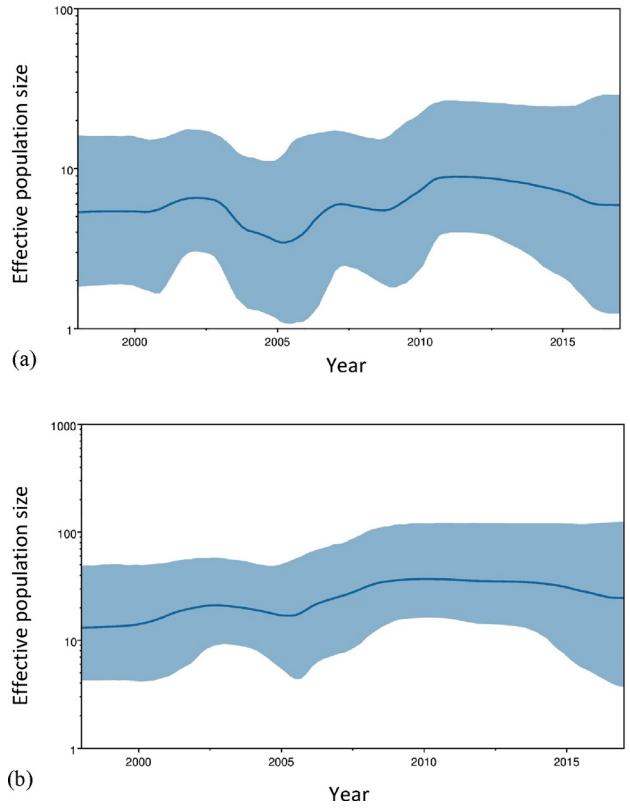

Fig. 2. Bayesian skyline plots of Iranian HA (a) and NA (b) sequences of H9N2 viruses since its first report. The dark solid line and the blue shaded region indicate effective population size and 95% highest posterior density interval, respectively. The vertical axis shows the effective population size and the horizontal axis indicates the isolation year of the samples

major changes in 1998–2017. There was only a gradual rise in the NA population from 2005 to 2017 (Fig. 2b).

Genetic analysis of the HA and NA genes

Deduced amino acid sequences of the HA and NA genes were aligned and compared with each other and other Iranian representatives retrieved from the GenBank (Supplementary Tables 1–2). All coding sequences of the analysed H9N2s consisted of 1,683 nucleotides (560 amino acids) except for one virus. A 12-nucleotide deletion resulting from the deletion of four amino acids at residues from 220 to 223 was observed in the FBM-1 virus. Although a 24-nucleotide deletion in HA1 was found in H7N2 viruses isolated from live bird markets in the United States (Suarez et al., 1999), this rare deletion had not been observed in H9N2 viruses before. Virulence molecular determinants of HA are mainly described by specific amino acid residues in the cleavage site and receptor binding site (RBS). In addition, the presence or absence of glycosylation sites around RBS plays a role in the pathogenicity of AI. Although the vaccine strain (Marand) had an RSSR motif at the cleavage site, all other H9N2 viruses isolated after 2013 shared a different motif (KSSR) at this site of the HA protein. Lack of multiple basic amino acids at the connecting peptide is regarded as a signature of LPAI (Steinhauer, 1999). By the analysis of RBSs, six substitutions were found in the studied isolates. Of these substitutions, Q226L in RBS, which are associated with binding preference to human-like α2,6-linked sialic acid receptors, were observed in nine recently isolated viruses. Thus, it can be stated that these viruses have zoonotic potential (Wan et al., 2008). The analysis of potential glycosylation sites with an NXS/T motif (where X represents any amino acid except proline) revealed that there were five (21, 94, 128, 285/289 and 292/296) and one (154) conserved sites in the HA1 and HA2 parts of the protein, respectively. In comparison with the vaccine strain, all nine H9N2s lost two potential glycosylation sites at amino acid residues 158 of HA1 and 219 of HA2. Furthermore, RIV-1 gained another glycan at position 133 of the HA1 portion and lost one at amino acid residue 213 of the HA2 part. The comparison of the antigenic sites of 10 Iranian H9N2s with reference to the previous studies (Kaverin et al., 2004; Peacock et al., 2016) revealed that recent H9N2s have seven alterations in these epitopes in comparison to the vaccine strain (Table 2). A deletion of amino acid residue 222 in the H9-A site was found in FBM-1 due to the deletion of four amino acid residues at positions 220–223. Moreover, mutation R172Q at the H9-B antigenic site was only observed in RIV-1 virus among the viruses analysed.

The complete NA genes of 10 H9N2s were determined and the coding sequence of all viruses encoded a protein of 469 amino acids. No stalk deletions at amino acids 38–39 and 63–65 were observed in the NA protein of H9N2 viruses. Pathogenicity indicators of NA are composed of enzyme active site, stalk length, haemadsorbing (HB) site and glycosylation sites. Analysis of active sites revealed that no mutation was found among the analysed viruses in known amino acid residues related to neuraminidase resistance. Analysis of three surface loops of the HB site in the NA protein revealed five substitutions (amino acid residues 366, 367, 372, 401 and 403) in two loops (370 and 400) of this site in the analysed viruses. A different pattern of glycosylation sites was observed; seven Iranian H9N2 viruses showed a similar pattern of glycosylation site, while three of them (Marand, FBM-3 and FBM-4) contained one more glycosylation in the NA protein. Moreover, recent isolates have lost a potential glycosylation site in position 70 compared to the vaccine strain.

DISCUSSION

Although vaccination against the H9N2 virus has been routinely adopted for two decades in Iran since the late 1990s, outbreaks of the disease occur with each passing year. H9N2s have a significant economic impact on poultry production throughout the Iranian industrial poultry sector. The currently used vaccine strains against H9N2 were originally derived from viruses back in 1998, when the first H9N2 virus was isolated in Iran (Bashashati et al., 2013). The aim of the present study was to perform sequencing and phylogenetic analysis of 10 viruses to determine the molecular evolution and genetic variation of the HA and NA genes of H9N2 viruses.

Table 2. Antigenic sites of HA genes of the Iranian H9N2 strains

Viruses	Antigenic site according to the study of Peacock et al. (2016)									Antigenic site according to the study of Kaverin et al. (2004)								
	H9-A					H9-B				Site I			Overlapping site			Site II		
	155	193	222	227	244	122	120[a]	149	172	135	157	162	133	189	198	145	193	226
Marand (98)	T	S	L	Q	R	Q	T	R	R	T	K	P	T	T	T	G	S	Q
EU477247 (99)	–[b]	N	–	–	–	–	–	–	–	–	–	–	–	–	–	D	N	L
EU477249 (00)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
EU477246 (02)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	Q
EF063726 (03)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
EF063730 (04)	–	–	–	–	–	–	–	–	M	–	R	–	–	–	–	–	–	L
EF063733 (05)	–	–	–	I	–	–	–	–	R	–	K	–	–	–	–	–	–	–
EU477245 (07)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
GU071968 (08)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
JX456178 (09)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
JN646748 (10)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
JQ970436 (11)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
JX294920 (12)	–	–	–	–	–	–	–	–	Q	–	–	–	–	–	–	–	–	–
FBM-1 (13)	–	–	–[c]	–	–	–	–	–	R	–	–	–	–	–	–	–	–	–
FBM-2 (14)	–	–	–	–	–	–	A	–	–	–	–	–	D	–	–	N	–	–
FBM-3 (14)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
FBM-4 (17)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
RIV-1 (17)	–	–	–	–	–	–	–	–	Q	–	–	–	N	–	–	–	–	–
RIV-2 (17)	–	–	–	–	–	–	–	–	R	–	–	–	D	–	–	–	–	–
RIV-3 (17)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
RIV-4 (17)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
RIV-5 (17)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

[a] This position was numbered according to H9 due to absence of this site in subtype H3.
[b] Dash indicates that the amino acid residues are the same as above.
[c] This site was deleted in FBM-1.

The topologies of HA and NA phylogenetic trees were very similar to each other and all the Iranian viruses fell into G1 sub-lineage in agreement with previous studies (Bashashati et al., 2013; Norouzian et al., 2014; Bahari et al., 2015). On the basis of phylogenetic analysis, the Iranian HAs are classified into four branches whereas NA genes fall into five categories. Recently studied H9N2 viruses were grouped into a cluster labelled '2014–2017 group' that shows the alteration of clusters over time. Moreover, the recent viruses show a distant genetic relationship at the nucleotide and amino acid levels (about 90%) with a common vaccine strain (Marand). BLAST analysis revealed that all tested strains were closely related to viruses (95–99%) isolated from Pakistan, which shares a border with Iran. Except for the FBM-1 virus, all other studied strains had a 1,683 nucleotides long coding sequence. Four amino acid deletions at positions 220–223 close to the RBS were found in the FBM-1 virus. This distinguishing characteristic was first reported in H7N2 viruses from live poultry markets in the northeast of the United States. This viral genotype showed eight amino acid deletions at positions 221–228 within the RBSs. Furthermore, the deletion of stalk in NA was detected in this genotype as it may compensate for the HA stalk deletion (Suarez et al., 1999). However, the studied isolates did not show any deletion in the stalk region of NA. In this regard, further investigations need to be conducted on the impact of this deletion on HA binding of the FBM-1 virus.

The demographic history of Iranian H9N2 viruses revealed that the diversification of H9N2 is increasing gradually over time. For the first three years after 1998, the effective population size of H9N2 viruses had a stable trend, perhaps because of the implementation of vaccination against H9N2. Although Iranian H9N2s have evolved under purifying selection, one amino acid residue at position 190 in the HA protein is under positive selection. An extensive study on selection pressures for all segments of Central Asian and Middle Eastern H9N2 viruses revealed that most coding positions were under purifying selection. Amino acid positions 160, 190 and 226 in the HA protein were detected to be under positive selection, located at the RBS, which may have an effect on receptor recognition (Fusaro et al., 2011).

Mutation of the amino acid residue at 226 in the RBS facilitates the transmission of AIV to mammalian species. HAs of avian viruses show a preference for binding to sialic acid α2,3-linked to galactose, while human viruses tend to use α2,6-linked sialic acid as abundant receptors in the upper respiratory tract (Matrosovich et al., 2009). Among the 10 analysed Iranian H9N2 isolates, 9 recently isolated viruses showed that the Q226L mutation, compared with the older strain (Marand), shows a preference to binding to α2,6-linked sialic acid (Obadan et al., 2019). This mutation plays a significant role in overcoming the host barrier

between avian and human, resulting in human infections with H9N2 viruses (Guo et al., 1999; Saito et al., 2001).

Five conserved amino acids (S367, S370, S372, N400 and W403) in the HB site were determined among all nine subtypes of NA with some exceptions (Varghese et al., 1997; Uhlendorff et al., 2009). Of five mutations in the 370 and 400 loops of the HB site, the analysed viruses possessed three substitutions in amino acid residues at positions 367, 372 and 403 in the conserved positions. Any mutation in the HB site of H9N2 viruses suggested the improvement of fitness in land-based poultry from aquatic birds as their natural reservoir (Matrosovich et al., 2001). Since the HB site plays a role in the replication of the virus in avian hosts, it is not known whether these substitutions can alter the pathogenicity of H9N2.

A comparison of recent H9N2s at antigenic sites with the vaccine strain exhibited a remarkable amino acid divergence. According to previous studies, of 17 determined antigenic sites by escape mutants (position 193 is common in two studies), seven substitutions occurred in recent H9N2 viruses (Kaverin et al., 2004; Peacock et al., 2016). Mutation in antigenic sites of the HA protein of H9N2s may produce antigenic variants and enable the virus to escape vaccine-induced neutralising antibodies, leading to vaccination failure (Peacock et al., 2016).

The results of the current study indicate that substitution in pivotal genetic markers of circulating field Iranian H9N2s continues to occur. Therefore, further studies are needed to evaluate the biological propensities of these mutations and assess the pathogenicity of circulating viruses. Although there have been reports on protection against clinical disease via vaccination, the H9N2 vaccine fails to protect from infection and virus shedding when antigenic variants are circulating (Lee et al., 2016; Xia et al., 2017). Accordingly, antigenic characterisation and systematic monitoring of prevailing H9N2s will be necessary to identify antigenically well-matched strains for a timely updating of the vaccine strain.

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SUPPLEMENTARY MATERIAL

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