

# Molecular characterization and phylogenetic analysis of human influenza A viruses isolated in Iran during the 2014–2015 season

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**Abstract** Influenza A viruses are an important cause of severe infectious diseases in humans and are characterized by their fast evolution rate. Global monitoring of these viruses is critical to detect newly emerging variants during annual epidemics. Here, we sought to genetically characterize influenza A/H1N1pdm09 and A/H3N2 viruses collected in Iran during the 2014–2015 influenza season. A total of 200 nasopharyngeal swabs were collected from patients with influenza-like illnesses. Swabs were screened for influenza A and B using real-time PCR. Furthermore, positive specimens with high virus load underwent virus isolation and genetic characterization of their hemagglutinin (HA) and M genes. Of the 200 specimens, 80 were influenza

A-positive, including 44 A/H1N1pdm09 and 36 A/H3N2, while 18 were influenza B-positive. Phylogenetic analysis of the HA genes of the A/H1N1pdm09 viruses revealed the circulation of clade 6C, characterized by amino acid substitutions D97N, V234I and K283E. Analysis of the A/H3N2 viruses showed a genetic drift from the vaccine strain A/Texas/50/2012 with 5 mutations (T128A, R142G, N145S, P198S and S219F) belonging to the antigenic sites A, B, and D of the HA protein. The A/H3N2 viruses belonged to phylogenetic clades 3C.2 and 3C.3. The M gene trees of the Iranian A/H1N1pdm09 and A/H3N2 mirrored the clustering patterns of their corresponding HA trees. Our results reveal co-circulation of several influenza A virus strains in Iran during the 2014–2015 influenza season.

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## Introduction

Influenza A viruses (IAVs) are a major cause of acute respiratory infections. Influenza outbreaks are associated with substantial health and economic burdens [1]. Influenza occurs globally with annual attack rates estimated at 5%–10% in adults and 20%–30% in children. Infection with IAV can result in hospitalization and death chiefly among high-risk groups (including individuals who are at the extremes of age, pregnant, immune compromised, or who have chronic underlying disease) [2]. Annual epidemics are estimated to result in about 3 to 5 million cases of severe illness and up to 500,000 deaths worldwide [2–4]. Seasonal influenza occurs in the winter months in countries with temperate climate, and its outbreaks usually last from 6 to 8 weeks in the community [2].

IAVs are enveloped viruses classified in the *Orthomyxoviridae* family. Their genome consists of eight

single-stranded, negative-sense RNA segments encoding for at least 11 proteins [5, 6]. IAV subtypes are distinguished based on the phylogenetic differences between the surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA) [7]. Since 1918, only three subtypes of influenza A viruses (H1N1, H2N2 and H3N2) have been known to form long-term stable lineages in humans [8].

IAVs evolve quickly because of their high substitutions rates and wide host range [9]. Matrix protein 2 (M2) ion channel blockers have been used to treat influenza infections for decades due to the pivotal role of this protein in viral replication and infection [10, 11]. However, the emergence of universal resistance to this group of antivirals has made them obsolete in clinical settings [12]. The HA protein is a major surface glycoprotein that plays important roles in virus attachment and entry [13]. Continuous accumulation of point mutations (antigenic drift) within the HA glycoprotein makes it necessary to routinely update the vaccine [14]. Antigenic shifts are mainly triggered by reassortment events, which result in the acquisition of antigenically novel HA glycoproteins and can cause pandemics. The first influenza pandemic of this century was proclaimed in April of 2009, with the emergence of a novel H1N1 IAV strain (A/H1N1pdm09) in Mexico and the USA and its subsequent global spread. This novel virus was a product of several previous reassortment events between avian, swine and human strains [15, 16].

Global monitoring of influenza viruses is critical to detect variants including vaccine-escape mutants, antiviral drug resistant variants, or antigenically shifted viruses. Here, we report the genetic diversity and evolution of the HA and MP genes of influenza A/H1N1pdm09 and A/H3N2 viruses circulating in Iran during 2014–2015.

## Materials and methods

### Clinical case definition

Two hundred nasopharyngeal swab specimens were collected during the 2014–2015 influenza season (from November 2014 to May 2015) from patients aged  $\geq 2$  months diagnosed with influenza-like symptoms (measured fever of  $\geq 38$  °C and cough; with onset within the last 7 days) [17] who sought medical attention at 2 outpatient clinics in Tehran, Iran. Patients with exudative pharyngitis or tonsillitis prior to presentation were excluded from our study.

The sample collection methodology was approved by the Research Ethics Committee of the Pasteur Institute of Iran and informed consents were obtained from patients prior to specimen collection. Collected swabs were placed

immediately in virus transport media tubes and frozen at  $-70$  °C until further analysis.

### RNA extraction and influenza virus detection

For detection of influenza virus, total RNA was extracted from the clinical specimens by using the High Pure Viral RNA Kit (Roche, Germany) according to the manufacturer's instructions. One step real-time RT-PCR was performed on a Corbett 6000 Rotor Gene system (Corbett, Victoria, Australia), according to the CDC protocol for identification of influenza A/H1N1pdm09, A/H3N2, and B viruses using a Superscript III platinum one step Quantitative RT-PCR kit (Invitrogen, Carlsbad, CA) [18, 19].

### Virus isolation

Influenza A positive specimens with high viral load (defined as threshold cycle "Ct" value  $\leq 20$ ) were selected for virus isolation. In brief, MDCK (Madin-Darby canine kidney) cells were seeded at a concentration of  $1.3 \times 10^5$  cells/ml in 24-well plates. Two days later, 200  $\mu$ l aliquots of the specimens were inoculated onto MDCK cells. The culture medium was then examined for hemagglutination activity using a 0.5% suspension of chicken erythrocytes. The viruses were passaged up to two times to obtain sufficient virus titers for RNA extraction and sequencing.

### Full-length PCR amplification of HA and M segments and sequencing

Viral RNA was extracted from the culture media of influenza isolates with sufficient hemagglutination titer using the YTA Viral Extraction Kit (YTA, Tehran, Iran), according to the protocol suggested by the manufacturer. The HA and M (matrix) genes were amplified using One-Step RT-PCR Kit (YTA, Tehran, Iran) with the primers listed in Table 1. Two primer sets specific for a highly conserved region of the M gene were used for A/H1N1pdm09 and A/H3N2 influenza virus. Two segment-specific primer sets (Table 1) were designed to amplify each of the A/H1N1pdm09 and A/H3N2 HA genes. PCR products were gel purified using GF-1 PCR Clean-up Kit (Vivantis, Malaysia) based on manufacturer's specifications and subjected to sequencing using an ABI sequence Genetic Analyzer (Applied Biosystems, Foster City, CA) at Sequence Laboratories of FirstBase company, Malaysia. The sequences were edited and assembled in Chromas Lite version 2.5.1 (Technelysium Pty Ltd., Australia) and CLC sequence viewer V.6.7. The nucleotide sequences of all samples were deposited in the GenBank database under the accession numbers shown in Table 2.

**Table 1** The sequences and binding positions of the primers used to amplify the coding regions of the M and HA genes of A/H1N1pdm09 and A/H3N2 subtypes, by one-step RT-PCR

Influenza subtype	Primer sequence (5'-3')	Target	Position (nt)	PCR product (bp)
H1N1	F: GATGAGTCTTCTAACCGAGG R: TTTACTCTAGCTCTATGTTGACA	M	25-44 986-1008	984
H1N1	F: AATGAAGGCAATACTAGTAGTTCTG R: CCAAATAGGCCTCTAGATTGAA	HA1	20-44 1042-1063	1044
H1N1	F: AAATTGAGACTGGCCACAGG R: CACTGTAGAGACCCATTAGAG	HA2	982-1001 1665-1685	704
H3N2	F: TATTGAAAGATGAGCCTTCTAA R: TTTACTCCAACCTCTATGCTGACA	M	17-38 986-1008	992
H3N2	F: GAGCTACATTCTATGTCTGG R: CTTAACATATCTGGGACAGG	HA1	21-40 959-978	958
H3N2	F: CATCACTCCAAATGGAAGCA R: TGTTGCACCTAATGTTGCC	HA2	891-910 1672-1690	800

**Table 2** A/H1N1pdm09 and A/H3N2 influenza isolates used in this study

Subtype	Strain	Collection date (DD/MM/YYYY)	Accession numbers	
			Segment 4 (HA gene)	Segment 7 (M gene)
A/H1N1pdm09	A/Iran/91755/2014	25/11/2014	KX571064	KX470422
	A/Iran/91874/2014	26/11/2014	KX571065	KX470423
	A/Iran/814/2014	01/12/2014	KT924264	KT984980
	A/Iran/802/2014	02/12/2014	KT924263	KT984979
	A/Iran/791/2014	03/12/2014	KT924262	KT984981
A/H3N2	A/Iran/91247/2014	22/11/2014	KX571067	KX470425
	A/Iran/91244/2014	25/11/2014	KX571066	KX470424
	A/Iran/92243/2014	01/12/2014	KX809569	KX827343
	A/Iran/92246/2014	03/12/2014	KX809570	KX827344
	A/Iran/116/2014	04/12/2014	KU182654	KP997231
	A/Iran/92172/2014	05/12/2014	KX809571	KX827345

To renumber HA sequences according to a cross-subtype numbering scheme, all protein sequences were BLASTed against the Burke Reference sequences [20] using the HA Subtype Numbering Conversion tool in the Influenza Research Database (IRD) system prior to sequence submission.

### Multiple sequence alignment and phylogenetic analysis

Evolutionary analyses based on HA and M gene sequences were carried out by including globally representative A/H1N1pdm09 and A/H3N2 sequences from the NCBI Influenza Resource Database (<http://www.ncbi.nlm.nih.gov/genomes/FLU>) and the Global Initiative on Sharing All Influenza Data (GISAID; <http://platform.gisaid.org/>) databases. All HA and M gene sequences were aligned by

using the ClustalW alignment tool in the Bioedit software (version 7.2.5). Phylogenetic trees were constructed by the Maximum likelihood method (ML) inferred on the basis of the best fit nucleotide substitution model for each of the M and HA genes as implemented in MEGA 6.0 [21]. The Hasegawa-Kishino-Yano model with a gamma distribution (HKY+G) was used for the HA gene and the Kimura 2-parameter model was used as the substitution model in M gene analysis. Initial trees for the heuristic search were obtained by applying the Neighbor-Joining and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. The reliability of the ML trees was evaluated by analyzing 1,000 bootstrap replicates and bootstrap values of >75% were considered significant. Designated clades were chosen based on the clustering patterns in the HA phylogeny.

## Results

### Surveillance and prevalence of influenza virus among patients with influenza-like illness (ILI)

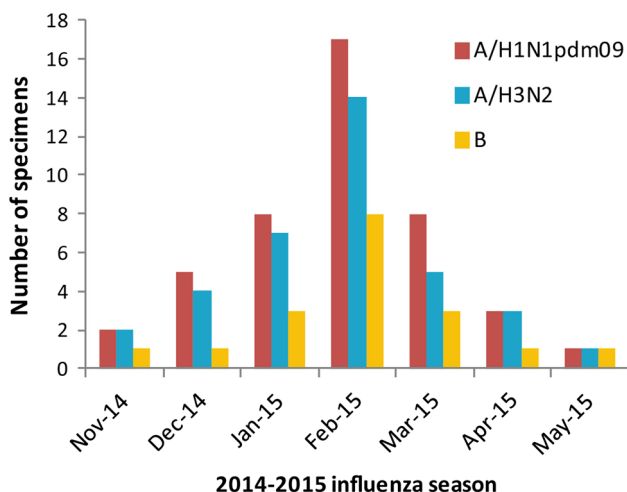
From November 2014 through May 2015, we collected nasopharyngeal swab specimens from 200 children and adults diagnosed with ILI. The median age of enrolled patients was 22 months (range, 2 months–70 years), and 181 specimens (90.5%) were from children. None of the patients sampled had previously been vaccinated against influenza. Ninety-eight (49%) patients were positive for influenza, among which, 80 (81.63%) were influenza A and 18 (18.37%) were influenza B. The influenza A specimens were further subtyped: 44 being A/H1N1pdm09 and 36 being A/H3N2. All three viruses co-circulated throughout the study period with a peak in February (Figure 1).

Twenty-three influenza A specimens (11 A/H1N1pdm09 and 12 A/H3N2) with low ( $\leq 20$ ) threshold cycle (Ct) value were selected for virus isolation. Eleven isolates (5 A/H1N1pdm09 and 6 A/H3N2) with an HA titer  $\geq 4$  were processed for further analysis.

### Phylogenetic trees of human influenza A strains in Iran

Phylogenetic trees of the HA genes were plotted to analyze the genetic diversity and relationships among our isolates and as a comparison with reference and vaccine strains (Supplementary Tables 1 and 2).

The HA phylogenetic tree of the Iranian A/H1N1pdm09 viruses isolated in this study is shown in Figure 2a. The HA genes of influenza A/H1N1pdm09 viruses have evolved into seven genetic groups and five subgroups (6A, 6B, 6C, 6B.1, and 6B.2) with



**Fig. 1** Distribution of A/H1N1pdm09, A/H3N2 and influenza B viruses in Iran from November 2014 through May 2015

A/California/07/2009 representing group 1 [22–25]. Phylogenetic analysis showed that the Iranian viruses belonged to two phylogenetic groups (6B and 6C) during 2014–2015. All of the viruses isolated in this study fell into genetic subgroup 6C, represented by A/Massachusetts/10/2013 and characterized by amino acid substitutions V234I and K283E in HA1 and E172K in HA2, compared with the representative virus of group 6 (A/St Petersburg/27/2011). One of the 5 Iranian isolates (A/Iran/791/2014) carried an additional substitution (A186T) in the HA1 subunit and clustered with the reference strain A/Ghana/DARI-0095/2014 in subgroup 6C.

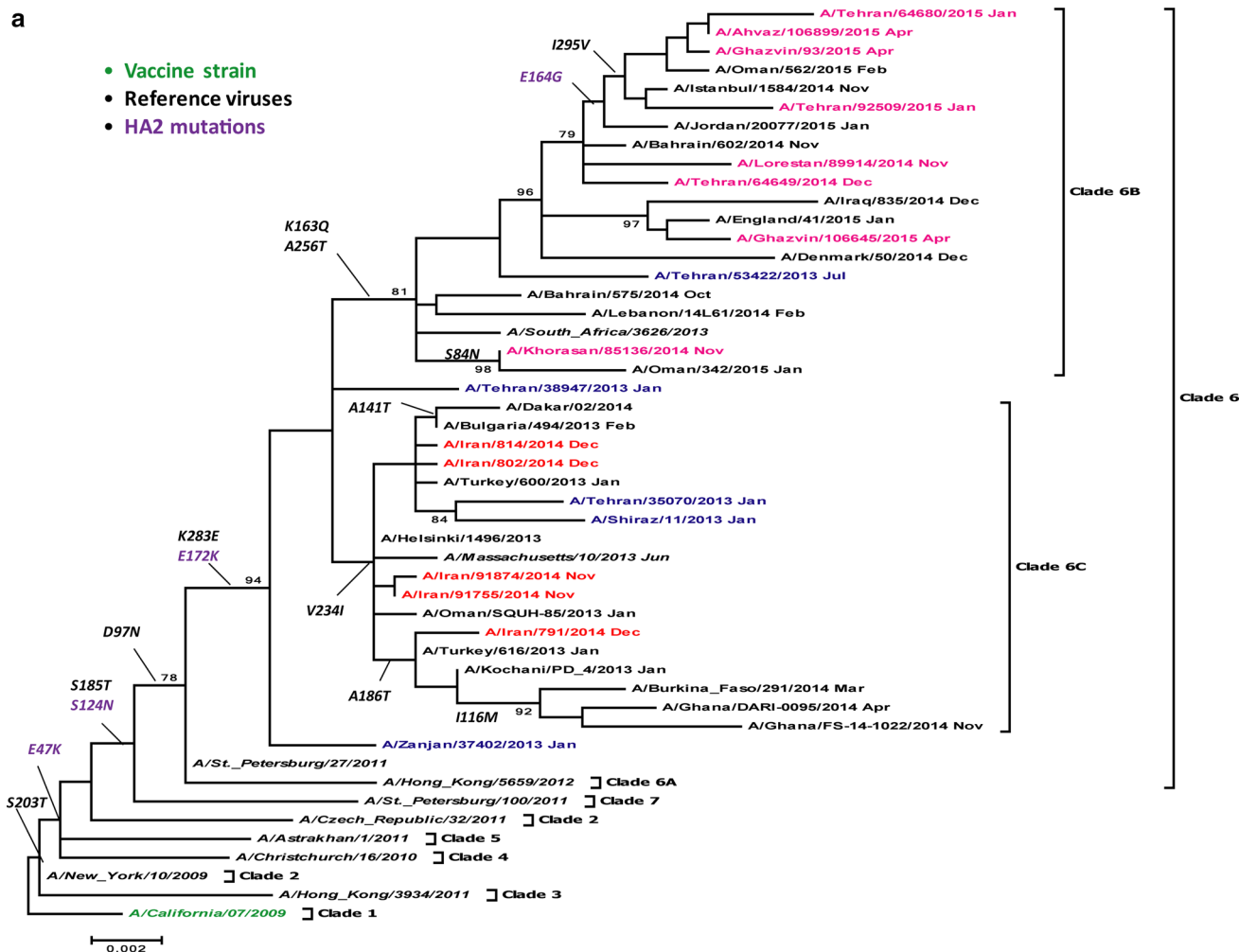
Viruses isolated in other parts of Iran during 2014 to 2015 clustered in subgroup 6B (represented by A/South Africa/3626/2013) that also harbored viruses from the Eastern Mediterranean Region (EMR) including Bahrain, Iraq, Jordan, and Lebanon. Viruses isolated in the previous season (2013–2014 influenza season) in Iran belonged to group 6 and clustered in the same subgroups with other Iranian A/H1N1pdm09 viruses isolated in the 2014–2015 influenza season (Figure 2a).

Comparison of the HA1 amino acid sequences of the 2014–2015 Iranian A/H1N1pdm09 isolates with the vaccine strain A/California/07/2009 revealed 98.40%–98.58% amino acid sequence similarity. Mutations S185T and S203T, located in the antigenic epitopes Sb and Ca, respectively, were observed among all five Iranian A/H1N1pdm09 isolates (Supplementary Table 1). The Iranian isolates also displayed amino acid mutations P83S, D97N, R223Q, V234I, K283E, and I321V in the non-antigenic sites of the HA1 subunit. Additional amino acid substitutions of E47K, S124N, and E172K were found in the HA2 region compared with A/California/07/2009.

Figure 2b shows the phylogenetic tree of representative A/H1N1pdm09 M genes. All five isolates belonged to the subgroup 6C of the M gene tree, similar to the HA tree. Amino acid substitutions in the M1 and M2 proteins are shown on the tree. All isolates retained the S31N substitution in the M2 ion-channel protein, known to confer resistance to adamantanes.

Analysis of the sequence homology of the M1 genes of the Iranian A/H1N1pdm09 viruses and the A/California/07/2009-vaccine strain revealed amino acid sequence identities of 98.41–98.80%, and the M2 genes shared protein identities of 98.10–98.96%. The Iranian isolates possessed a V80I mutation in the M1 protein and a D21G mutation in the M2 protein.

The HA phylogenetic tree of the Iranian A/H3N2 viruses isolated in this study is shown in Figure 3a. Seven discrete clades of H3N2 (labeled clades 1–7) and several subclades have evolved during the past few years and are circulating globally [26–28]. These clades are classified



**Fig. 2** Phylogenetic trees constructed on the basis of the HA (1701 nucleotides) (a) and M genes (982 nucleotides) (b) of the 5 H1N1pdm09 influenza viruses collected in Iran from 2014 to 2015. The trees were constructed using the maximum likelihood (ML) method with bootstrap analysis of 1,000 replicates. A/California/07/2009 was used as the root for the tree and bootstrap values greater than 75% are shown. Amino acid mutations characteristic of the main clusters or the Iranian isolates are indicated on the branches. The

vaccine strain is in bold green and reference strains are in bold black. The representative strain of each clade is shown in italics. Iranian isolates from 2014-2015 season are shown in red and isolates from 2013-2014 season are shown in blue. Viruses isolated in other parts of Iran in 2014 to 2015 are highlighted in pink. In the M tree, only reference samples for which the M gene sequence was available in the GenBank database are included

following the CDC nomenclature [29] and are defined based on the HA nucleotide phylogeny [30]. The HA genes of the 6 Iranian isolates belonged to two subclades. Two viruses belonged to the clade 3C.2 (represented by A/Hong Kong/146/2013) along with viruses from the EMR including Egypt, Iraq, and Jordan. One of these isolates (A/Iran/116/2014), clustered with the reference influenza A/H3N2 strain (A/Hong Kong/5738/2014) belonging to genetic subgroup 3C.2a. Viruses in this subgroup carry the 3C.2 amino acid substitutions plus L3I, N144S, F159Y, K160T, N225D and Q311H in HA1 substitutions [23, 31]. Four other isolates clustered within clade 3C.3 viruses (represented by A/Samara/73/2013) along with viruses from Bahrain, Kuwait, and Lebanon. One of these isolates

(A/Iran/92172/2014), clustered with the A/Netherlands/525/2014 reference strain in genetic subgroup 3C.3b. Isolates belonging to this subgroup possess E62K, K83R, N122D, L157S and R261Q substitutions in HA1 and M18K in HA2, in addition to those characteristic of clade 3C.3 [23, 31]. All of the viruses isolated in other parts of Iran during 2014 to 2015 clustered in subgroup 3C.2a. Viruses isolated in the previous season (2013-2014 season) in Iran belonged to two different groups. Two viruses belonged to clade 3C.3 and clustered in the same subgroup as the 2014-2015 Iranian A/H3N2 viruses isolated in this study. Three other viruses from the 2013-2014 season clustered in clade 1 and were closely related to the 2012 A/Perth/16/2009 vaccine virus. The 2013-2014 viruses



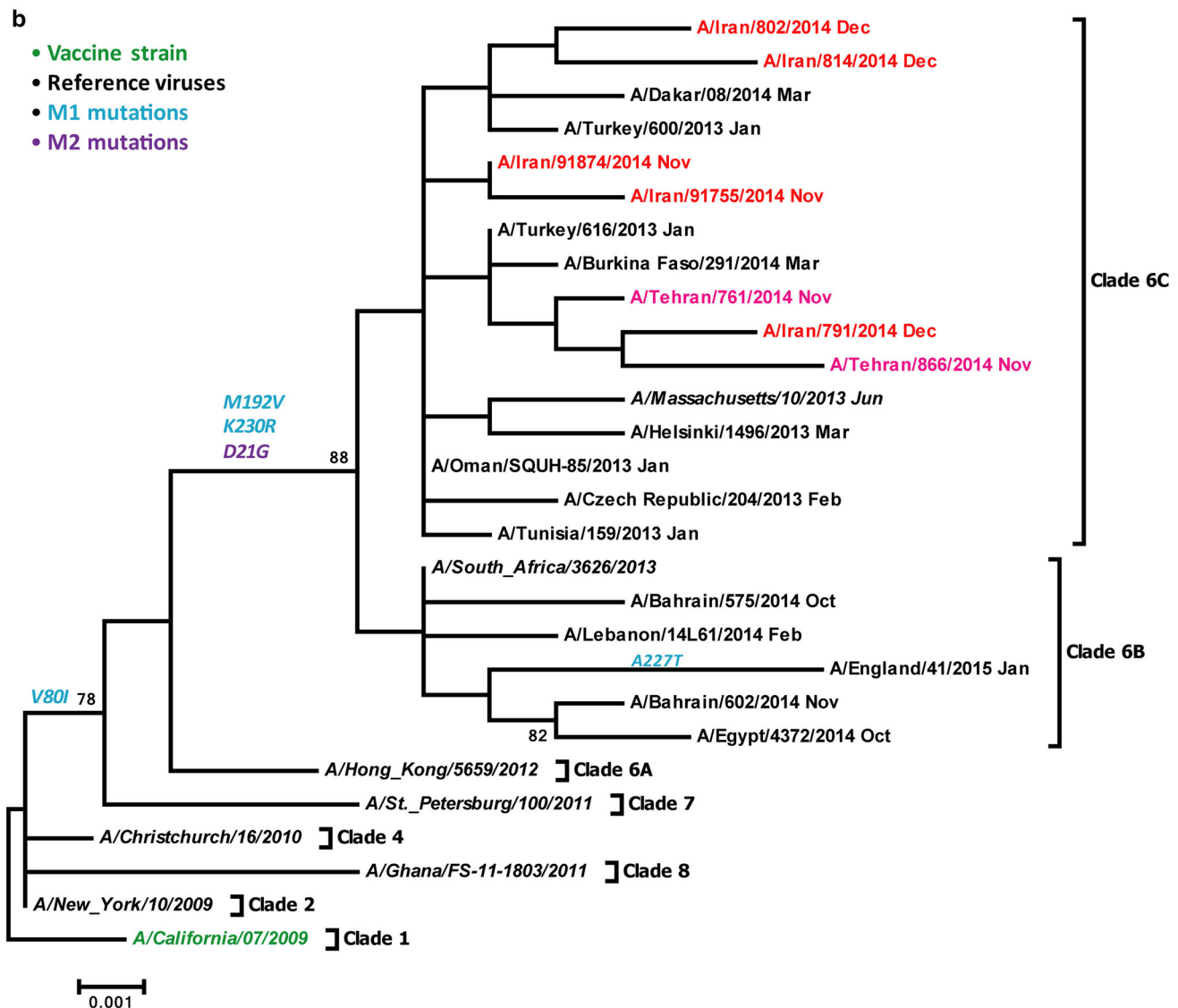


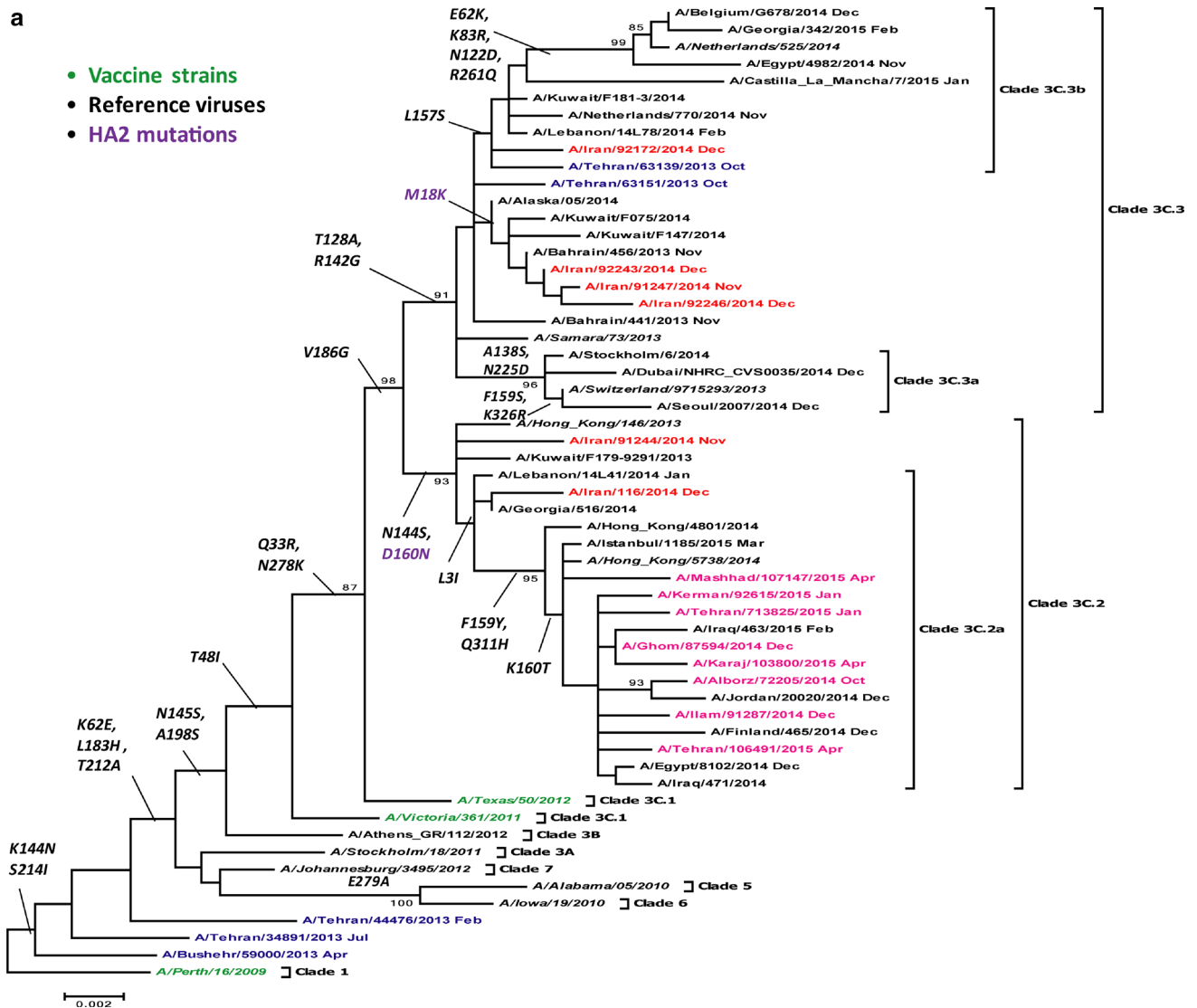
Fig. 2 continued

displayed a drift relative to the 2013-2014 vaccine strain, A/Victoria/361/2011, which belongs to clade 3C.1.

The A/H3N2 viruses from this study possessed an N145S mutation (antigenic site A) and T128A and P198S mutations (antigenic site B) in comparison with A/Texas/50/2012 vaccine strain (Supplementary Table 2). Four isolates carried substitution R142G in antigenic site A and 1 isolate had an S219F mutation, located in antigenic site D. Additional amino acid substitutions, L3I and K264R, were found in the non-antigenic sites of the HA1 subunit compared to A/Texas/50/2012. Notably, the Iranian A/H3N2 isolates displayed a drift relative to the 2014-2015 vaccine strain, A/Texas/50/2012, which belongs to clade 3C.1. Thus, the Iranian viruses possessed 3 to 4 amino acids mutations at

key antigenic sites of the HA protein compared to the vaccine strain, indicating a potential vaccine mismatch.

Figure 3b shows the phylogenetic tree of the M genes of representative viruses with the corresponding HA genetic groups indicated. The Iranian isolates were accommodated in clades 3C.2 and 3C.3 in the M tree, similar to their topologies within the HA tree. All viruses retained the S31N substitution in the M2 ion-channel protein. Other mutations in the M1 and M2 proteins are shown on the tree (Figure 3b). Analysis of the sequence homology of the M1 genes of Iranian A/H3N2 viruses and the A/Texas/50/2012 vaccine strain revealed amino acid sequence identities of 99.20- 99.60%, and the M2 genes shared protein identities of 96.90- 98.96%.



**Fig. 3** Phylogenetic trees constructed on the basis of the HA (1701 nucleotides) (a) and M genes (982 nucleotides) (b) of the 6 H3N2 influenza viruses collected in Iran from 2014 to 2015. The trees were constructed using the maximum likelihood (ML) method with bootstrap analysis of 1,000 replicates. A/Perth/16/2009 was used as the root for the tree and bootstrap values greater than 75% are shown. Amino acid mutations characteristic of the main clusters or the Iranian isolates are indicated on the branches. Vaccine strains are in

bold green and reference strains are in bold black. The representative strain of each clade is shown in italics. Iranian isolates from 2014-2015 season are shown in red and isolates from 2013-2014 season are shown in blue. Viruses isolated in other parts of Iran in 2014 to 2015 are highlighted in pink. In the M tree, only reference samples for which the M gene sequence was available in the GenBank database are included

### Discussion

Human influenza viruses continuously circulate around the world and cause seasonal “flu” epidemics each year. Iran is the second largest country in the EMR and hosts a large number of travelers and immigrant workers who come from other countries, including neighboring countries. Therefore, monitoring influenza in Iran is critical to improve our understanding of influenza patterns in this region.

Outbreaks of human influenza usually occur during the winter months in the northern hemisphere temperate region but the exact time of onset and duration of the influenza season varies by country and by year [32]. In most countries of the Middle East and North Africa, the timing (beginning and peak) of the 2014-2015 season was the same as previous seasons [32]. Our data showed that influenza activity in Iran during the 2014-2015 season peaked in February, similar to Algeria, Georgia, and Tunisia. While in other countries like Lebanon, Iraq, Palestine, Turkey,

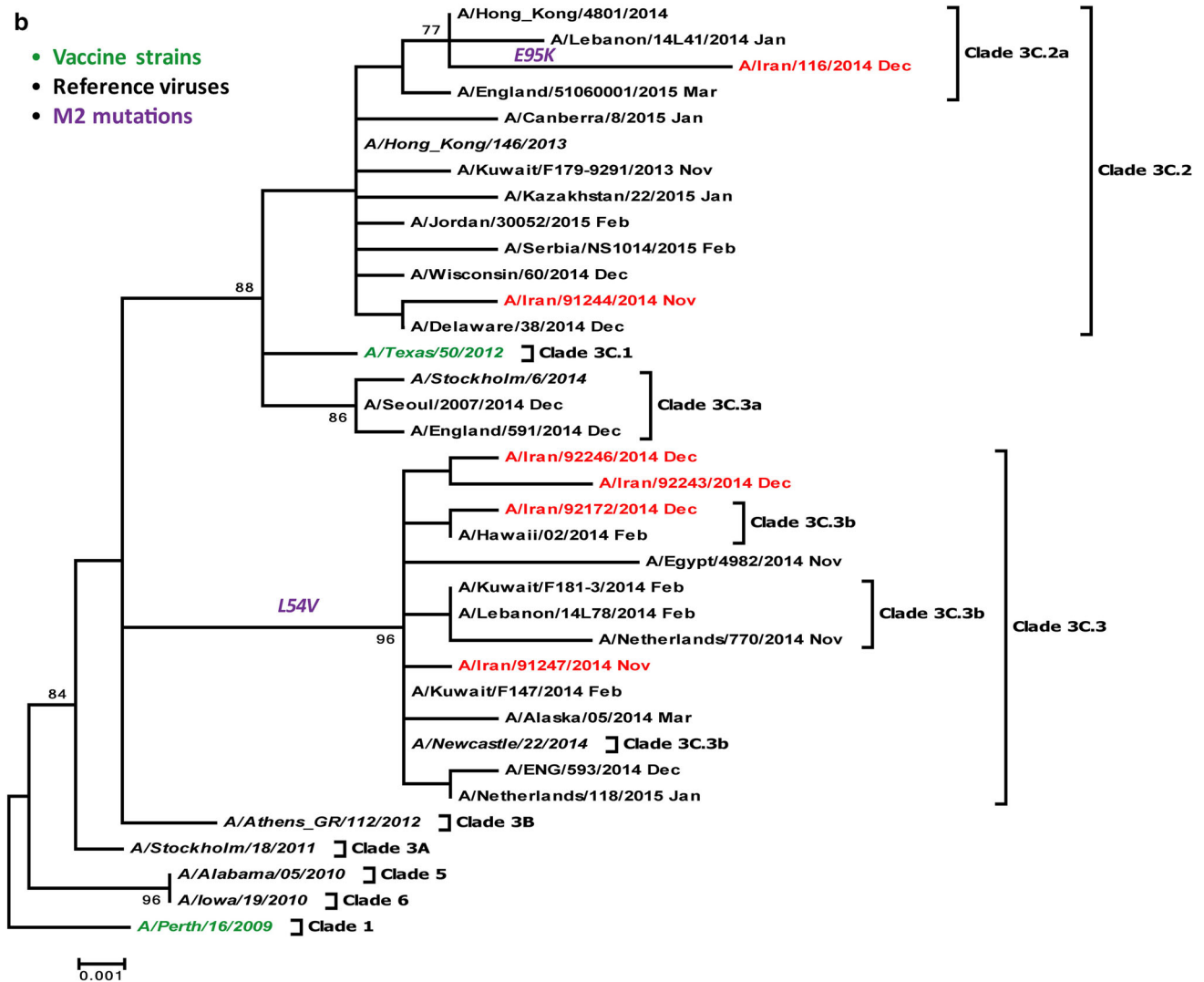


Fig. 3 continued

Jordan, and Kuwait, influenza activity peak was delayed till March or April [32, 33].

According to a WHO report, most countries throughout the Middle East and North Africa experienced a lower level of influenza activity during the 2014–2015 season than in the previous 2 seasons, i.e. 2012–2013 and 2013–2014 [32]. In the 2014–2015 season, influenza A/H1N1pdm09, A/H3N2, and influenza B viruses were detected in the Middle East and North Africa but the predominant virus varied by country. Influenza A/H1N1pdm09 was predominant in Algeria, Bahrain, Jordan, Kuwait, Libya, and Turkey, while influenza B was predominant in Lebanon and Georgia [32, 33]. The influenza A/H1N1pdm09 subtype was detected as the predominant virus during the 2014–2015 influenza season in Iran with co-circulation of A/H3N2 and B viruses. Influenza A/H1N1pdm09 viruses accounted for 55% and A/H3N2 accounted for 45% of the

IAVs detected between November 2014 and May 2015 in Iran.

The HA protein is a major antigenic target and its gene exhibits a high degree of variation [34]. Sequence analysis of the HA genes of A/H1N1pdm09 viruses demonstrated that most of the recently circulating viruses belonged to the genetic subgroup 6B [35]. In Iran, we detected circulation of clades 6B and 6C during the 2014–2015 season.

Globally, influenza A/H3N2 viruses collected from September 2014 to January 2015 fell into clades 3C.2 and 3C.3 [35]. Viruses in sub-clade 3C.2a became predominant in many regions of the world, except parts of Asia, and parts of Eastern Europe and Africa where subclade 3C.3a viruses predominated. In addition, 3C.3 and sub-clade 3C.3b viruses were still in circulation [32]. Similarly, A/H3N2 clades 3C.2 and 3C.3 were detected in Iran during the 2014–2015 season. One out of the six studied isolates



belonged to subgroup 3C.2a and four isolates clustered in 3C.3 and subgroup 3C.3b during the related season. Our results were in accordance with the data presented by WHO in 2014–2015 influenza season in the northern hemisphere.

For the 2014–2015 season, the recommendations for the influenza trivalent vaccine included an A/California/07/2009 (H1N1pdm09)-like virus and an A/Texas/50/2012 (H3N2)-like virus. The circulating A/H1N1pdm09 viruses were both antigenically and genetically related to the selected vaccine virus for the 2014–2015 season [32]. HA genes of the Iranian A/H1N1pdm09 isolates possessed eight amino acid changes compared to the vaccine strain A/California/07/2009. Mapping the substitutions to known HA antigenic sites, revealed an S185T and an S203T within the Sb and Ca epitopes, respectively, of the H1 HA protein. These amino acid substitutions are located within the HA receptor-binding domain (RBD) and may potentially influence HA antigenicity and virulence [36, 37]. The S185T substitution has been shown to affect receptor-binding affinity of the A/H1N1pdm09 viruses [37, 38]. Mutation S203T affects the infectivity and transmissibility of influenza A/H1N1pdm09 virus in humans [39].

In the 2014–2015 season, a significant proportion of circulating A/H3N2 viruses were antigenically drifted from the A/Texas/50/2012 (H3N2)-like vaccine viruses [28, 35]. The majority of A/H3N2 viruses were antigenically similar to A/Switzerland/9715293/2013, the strain selected in September 2014 for the 2015 Southern hemisphere vaccine and in February 2015 for the 2015–2016 Northern hemisphere vaccine [32]. Phylogenetic analysis of the Iranian A/H3N2 viruses showed that the 2014–2015 isolates were genetically divergent from the vaccine strain (A/Texas/50/2012), similar to those circulating in other countries during the same season [35]. The A/H3N2 Iranian isolates had 3–4 mutations belonging to antigenic site A (R142G, N145S), B (T128A, P198S), and D (S219F), which explains the suboptimal protection afforded by the latter vaccine [40, 41].

In conclusion, the results of this study contribute to our understanding of the overall genetic evolution of influenza A/H1N1pdm09 and A/H3N2 viruses. Further studies characterizing a larger number of viruses over multiple seasons are needed to fully elucidate influenza activity and circulation patterns in Iran and the EMR in general.

**Author contributions** All authors contributed extensively to the work presented in this paper.

**Compliance with ethical standards**

**Conflict of interest** The authors declare that they have no conflict of interest.

**Ethical approval** All procedures performed in studies involving human participants were in accordance with the ethical standards of the Pasteur's institutional research committee of Iran and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

**Informed consent** Informed consent was obtained from all individual participants included in the study.

## References

1. Medina RA, García-Sastre A (2011) Influenza A viruses: new research developments. *Nat Rev Microbiol* 9:590–603
2. World Health Organization Influenza (seasonal) (2015) Fact sheet N 211. April 2009. <http://www.who.int/mediacentre/factsheets/fs211/en>. Accessed 23 Apr 2015
3. Nguyen-Van-Tam JS, Hampson AW (2003) The epidemiology and clinical impact of pandemic influenza. *Vaccine* 21:1762–1768
4. World Health Organization Influenza (seasonal) (2016) Fact sheet. March 2014. <http://www.who.int/mediacentre/factsheets/fs211/en>. Accessed 23 June 2016
5. Webster RG, Bean WJ, Gorman OT, Chambers TM, Kawaoka Y (1992) Evolution and ecology of influenza A viruses. *Microbiol Rev* 56:152–179
6. Wright PF, Webster RG (2001) Orthomyxoviruses. *Fields Virol* 1:1533–1579
7. Tong S, Zhu X, Li Y, Shi M, Zhang J, Bourgeois M, Yang H (2013) New world bats harbor diverse influenza A viruses. *PLoS Pathog* 9:e1003657
8. Yoon S-W, Webby RJ, Webster RG (2014) Evolution and ecology of influenza A viruses. In: *Influenza pathogenesis and control*, vol I. Springer, New York, pp 359–375
9. Holmes EC (2010) Helping the resistance. *Science* 328:1243–1244
10. Gu R, Liu LA, Wei D (2014) Drug inhibition and proton conduction mechanisms of the influenza A M2 proton channel. *Adv Exp Med Biol* 205–226
11. Bouvier NM, Palese P (2008) The biology of influenza viruses. *Vaccine* 26:D49–D53
12. Dong G, Peng C, Luo J, Wang C, Han L, Wu B, Ji G (2015) Adamantane-resistant influenza A viruses in the world (1902–2013): frequency and distribution of M2 gene mutations. *PLoS One* 10:e0119115
13. Li W, Shi W, Qiao H, Ho SYW, Luo A, Zhang Y, Zhu C (2011) Positive selection on hemagglutinin and neuraminidase genes of H1N1 influenza viruses. *Virol J*. doi:10.1186/1743-422x-8-183
14. Hay AJ, Gregory V, Douglas AR, Lin YP (2001) The evolution of human influenza viruses. *Philos Trans R Soc Lond B Biol Sci* 356:1861–1870
15. Mir MA, Lal RB, Sullender W, Singh Y, Garten R, Krishnan A, Broor S (2012) Genetic diversity of HA1 domain of hemagglutinin gene of pandemic influenza H1N1pdm09 viruses in New Delhi, India. *J Med Virol* 84:386–393
16. Scalera NM, Mossad SB (2009) The first pandemic of the 21st century: review of the 2009 pandemic variant influenza A (H1N1) virus. *Postgrad Med* 121:43–47
17. World Health Organization (2013) Global epidemiological surveillance standards for influenza. Edited by Influenza WHO/WGESSf. World Health Organization, Geneva. [http://www.who.int/influenza/resources/documents/WHO\\_Epidemiological\\_Influenza\\_Surveillance\\_Standards\\_2014.pdf](http://www.who.int/influenza/resources/documents/WHO_Epidemiological_Influenza_Surveillance_Standards_2014.pdf)

18. Centers for Disease Control and Prevention (2009) CDC protocol of realtime RTPCR for influenza A (H1N1). [http://www.who.int/csr/resources/publications/swineflu/CDCRealtimeRTPCR\\_SwineH1Assay-2009\\_20090430.pdf](http://www.who.int/csr/resources/publications/swineflu/CDCRealtimeRTPCR_SwineH1Assay-2009_20090430.pdf)
19. Han Y, Sun N, Lv Q-Y, Liu D-H, Liu D-P (2016) Molecular epidemiology and phylogenetic analysis of HA gene of influenza A (H1N1) pdm09 strain during 2010–2014 in Dalian, North China. *Virus Genes* 1–7
20. Burke DF, Smith DJ (2014) A recommended numbering scheme for influenza A HA subtypes. *PLoS One* 9:e112302
21. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol Biol Evolut* 30:2725–2729
22. Nelson M, Spiro D, Wentworth D, Fan J, Beck E, George KS, Hine E et al (2009) The early diversification of influenza A/H1N1pdm. *PLoS Curr Influenza*
23. European Centre for Disease Prevention and Control (ECDC) (2014) Influenza virus characterisation, summary Europe, November 2014. ECDC, Stockholm. <http://www.ecdc.europa.eu/en/publications/Publications/ERLI-Net-report-November-2014.pdf>
24. European Centre for Disease Prevention and Control (2013) Influenza virus characterisation, summary Europe, December 2013. ECDC, Stockholm. <http://ecdc.europa.eu/en/publications/publications/influenza-virus-characterisation-dec-2013.pdf>
25. Broberg E, Melidou A, Prosenc K, Bragstad K, Hungnes O (2016) Predominance of influenza A (H1N1) pdm09 virus genetic subclade 6B. 1 and influenza B/Victoria lineage viruses at the start of the 2015/16 influenza season in Europe. *Eurosurveillance* 21
26. European Centre for Disease Prevention and Control (ECDC) (2012) Influenza virus characterization, summary Europe, March 2012. In: Technical document. Community network of reference laboratories for human influenza in Europe. <http://ecdc.europa.eu/en/publications/Publications/1204-TED-CNRL-report.pdf>. Accessed 7 Dec 2013
27. European Centre for Disease Prevention and Control (ECDC) (2012) Influenza virus characterization, summary Europe, June 2012. In: Technical document. Community network of reference laboratories for human influenza in Europe. <http://www.ecdc.europa.eu/en/publications/Publications/Influenza-visus-characterisation-June-2012.pdf>. Accessed 7 Dec 2013
28. Broberg E, Snacken R, Adlhoch C, Beauté J, Galinska M, Perelyaslov D (2015) Start of the 2014/15 influenza season in Europe: drifted influenza A (H3N2) viruses circulate as dominant subtype. *Eurosurveillance* 20
29. Cox N (2012) Seasonal influenza and zoonotic influenza. In: Information for the vaccine and related biological products advisory committee meeting. Silver Spring, Maryland, 28 Feb 2012
30. Stucker K, Schobel S, Olsen R, Hodges H, Lin X, Halpin R, Das S et al (2015) Haemagglutinin mutations and glycosylation changes shaped the 2012/13 influenza A (H3N2) epidemic, Houston, Texas. *Euro Surveill* 20:21122
31. McCauley John DR, Lin YP, Zheng X (2015) Report prepared for the WHO annual consultation on the composition of influenza vaccine for the Southern Hemisphere. MRC National Institute for Medical Research, London
32. World Health Organization (2015) Review of the 2014–2015 influenza season in the northern hemisphere. *Wkly Epidemiol Rec* 90:281–296
33. Saito R, Akinobu H, Shaker R, Akel I, Assaf-Casals A, Lteif M, Odagiri T (2016) Characterization of influenza outbreaks in Lebanon during the 2013/14 and 2014/15 seasons. *East Mediterr Health J* 22:547
34. Ferguson NM, Galvani AP, Bush RM (2003) Ecological and immunological determinants of influenza evolution. *Nature* 422:428–433
35. World Health Organization (2015) Recommended composition of influenza virus vaccines for use in the 2015–2016 northern hemisphere influenza season. *Wkly Epidemiol Rec* 90:97–108
36. Caton AJ, Brownlee GG, Yewdell JW, Gerhard W (1982) The antigenic structure of the influenza virus A/PR/8/34 hemagglutinin (H1 subtype). *Cell* 31:417–427
37. Sriwilajaroen N, Suzuki Y (2012) Molecular basis of the structure and function of H1 hemagglutinin of influenza virus. *Proc Jpn Acad Ser B* 88:226–249
38. Koel BF, Mögling R, Chutinimitkul S, Fraaij PL, Burke DF, van der Vliet S, Osterhaus AD et al (2015) Identification of amino acid substitutions supporting antigenic change of influenza A (H1N1) pdm09 viruses. *J Virol* 89:3763–3775
39. Pan C, Cheung B, Tan S, Li C, Li L, Liu S, Jiang S (2010) Genomic signature and mutation trend analysis of pandemic (H1N1) 2009 influenza A virus. *PLoS One* 5:e9549
40. Chambers BS, Parkhouse K, Ross TM, Alby K, Hensley SE (2015) Identification of hemagglutinin residues responsible for H3N2 antigenic drift during the 2014–2015 influenza season. *Cell Rep* 12:1–6
41. Webster R, Laver W (1980) Determination of the number of nonoverlapping antigenic areas on Hong Kong (H3N2) influenza virus hemagglutinin with monoclonal antibodies and the selection of variants with potential epidemiological significance. *Virology* 104:139–148