Antigenic Variation of the Haemagglutinin Gene of the Influenza A (H1N1) pdm09 Virus Circulating in Shiraz, February-April 2013

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ABSTRACT

Background: A new pandemic influenza A (H1N1) emerged in April 2009, causing considerable morbidity and mortality. Since mutations in the haemagglutinin (HA) may influence the antigenicity and pathogenicity of the virus, continued epidemiological and molecular characterization for the effective control of pandemic flu and developing of more appropriate vaccine is crucial. **Objective:** To monitor the molecular evolution of A (H1N1) pdm09 viruses in a specific time period in Shiraz, Southern Iran. **Methods:** A total of 200 samples were collected from February-April 2013. HA gene of the isolates was amplified and sequenced. Phylogenetic analysis of the HA gene was performed. **Results:** Out of 200 samples, a total of 77 (38.5%) samples were confirmed as A (H1N1) pdm09 virus using Real-time PCR method. Nucleotide similarity of our study strains with respect to reference strain A/California/07/2009 (H1N1) was 97.5%-98.5%. Phylogenetic analysis of our study strains indicated that the dominant A (H1N1) pdm09 clade was clade 7 and the dominant genetic group in circulating strains in Shiraz was genetic group 6. Some of our study strains showed substitutions at or in the vicinity of the antigenic sites of the HA1 region which may affect the efficacy of the vaccine. **Conclusion:** Our study strains showed a high homology to the vaccine strain. Our findings confirm the genetic variability of influenza A (H1N1) pdm09 and highlight the necessity of continuous molecular study of the virus for effective management of influenza.

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Keywords: Antigenic Site, Haemagglutinin, Influenza A (H1N1) pdm09, Vaccine

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INTRODUCTION

Influenza is one of the major threats to the public health (1). On average, three to five million people suffer from severe infection with influenza viruses world-wide; of these, half a million die of influenza annually (2,3).
Influenza viruses are negative strand RNA viruses belonging to the Orthomyxoviridae family (4). Based on the differences in the structural (M, NP) genes, influenza A is classified into three types (A, B and C). Influenza A viruses are divided into subtypes according to antigenic properties of surface glycoproteins, haemagglutinin (HA), and neuraminidase (NA) (5,6).
Influenza viruses are subjected to high frequency of point mutations (antigenic drift) and the assortment (antigenic shift) of external glycoprotein genes, haemagglutinin (HA) and neuraminidase (NA) genes, leading to antigenic changes which cause epidemics, or pandemic respectively (7).
A new influenza A (H1N1) virus pandemic emerged in Mexico and the United States in March 2009 (8).The virus spread in all continents rapidly and still is one of the circulating influenza viruses in humans (9,10).
Indeed, (H1N1) pdm09 virus is a recent reassortant of gene segments from avian, human and swine lineages. Phylogenetic analysis proved that NA and M genes arose from avian-like Eurasian swine H1N1 lineage, HA, NP and NS genes from classical swine virus lineage, PB2 and PA genes from North American avian viruses lineage and the PB1 gene from human H3N2 virus lineage (7,11).
High human to human transmission of A (H1N1) pdm09 virus may be due to the absence of pre-existing immunity in humans (10,12), although it should be noted that Influenza A (H1N1) pdm09 virus shows differences in tropism, replication, transmission, and pathogenesis in comparison with seasonal H1 and H3 strains (13).
The first laboratory-confirmed influenza A (H1N1) pdm09 case in Fars province was identified on 15 July 2009 and until 15 December 2009, influenza of A (H1N1) pdm09 was reported in 297 suspected patients with influenza (14). As December 30, 2009, 3672 cases of H1N1 flu with 140 deaths have been reported in Iran (15). Since mutations in the haemagglutinin (HA) may alter receptor binding specificity and pathogenicity of circulating strains (7,10), continued epidemiological and molecular studies are essential for monitoring the modifications in virus genome, affecting the pathogenicity and identification of emerging variants. Such surveillances lead to effective management of pandemic flu and development of more appropriate vaccines to control influenza A (H1N1) pdm09 virus infection.
The purpose of this study was to analyze the prevalence and molecular and phylogenetic analysis of new influenza A/H1N1 (pdm09) virus strains that circulated in Fars province from February 2013 to April 2013, and to identify the sequence variations in comparison with vaccine strain and circulating viruses in other regions of the world.

MATERIALS AND METHODS

Sample Collection. A total of 200 samples from patients with Influenza-like-illness (ILI) showing symptoms including fever, myalgia cough and sore throat, were collected by outpatient hospital clinics from February 2013 to April 2013. Patients with other

viral infections were excluded. Nasopharyngeal samples were immediately placed in viral transport media tubes and stored at -80 until further processing.

RNA Extraction and Real-Time RT-PCR for Detection of H1N1 (pdm09) Virus. RNA was extracted from 200 µl of the clinical specimens that were placed in transport medium using the high pure viral RNA extraction kit (Roche, Berlin, Germany). Extracted RNAs were stored at -80°C until further steps.

Real-time RT-PCR was performed using superscript III platinum one step Quantitative RT-PCR kit (Invitrogen, Carlsbad, CA, USA). In this reaction, 4 µl of extracted RNA combined with 16 µl of the master mix comprised10 µl 2x reaction mix, 5 µl RNase-DNase free water and 0.4 µl of each primer and superscript III RT/platinum Taq mix with the volumes of 40µl and 10µl respectively.

Based on the CDC real-time RT-PCR protocol (2009), reverse transcription was achieved at 50°C for 30 min, followed by enzyme inactivation at 95°C for 2 min and PCR steps included 45 cycles at 95°C for 15 s and 55°C for 30s(16). Real-time PCR was done on the Corbett 6000 Rotor Gene system (Corbett, Victoria, Australia).

Virus Isolation. Since titer of the viruses was low, in order to increase titer of the viruses, 200 micro liters suspension of each positive sample was inoculated onto confluent Madin-Darby-Canine Kidney (MDCK) cell line grown in Dulbeco´s Modified Eagle̛s Medium (DMEM) supplemented with 10% fetal bovine serum, 100 µg/ml streptomycin, 100 units/ml penicillin G and 2 mg/ml trypsin. The infected cells were incubated at 35°C. The culture medium was investigated daily for cytopathic effect and then confirmed by haemagglutinin activity (HA) test using guinea pig erythrocytes (17). Then, RNA of the supernatant of the cell culture was extracted.

cDNA Synthesis and Conventional PCR for the Amplification of HA Genes. Extracted RNA from positive HA supernatant was subjected to RT-PCR. cDNA synthesis was conducted using vivantis kit using manufacturer's instruction. To amplify the whole coding region of the HA gene, PCR with specific primers (18) was carried out. PCR products were purified and each DNA product was verified following electrophoresis on 2% agarose gel. Purified preparations of the HA genes of 9 randomly selected samples were sequenced and analyzed by the CLC sequence viewer and Bio Edit softwares.

Phylogenetic Analysis. Molecular phylogenetic analysis was performed for all 9 strains with reference to the HA genes of the 83 global isolates available in Gen Bank (19,20). The tree was constructed with MEGA version 6, using neighbor-joining (NJ) method with 1000 bootstrap replicates.

RESULTS

From Feb 2013 to April 2013, a total of 77 samples (38.5%) were found positive for influenza A (H1N1) pdm09 virus using Real-Time PCR method. Also 20 samples (10%) were found positive for influenza A (H3N2) virus using RT-PCR. The patients ranged in age from 1 year to 70 years and there were 42 males and 35 females (Table 1). All sampled patients had mild disease and none of them needed hospitalization. HA sequences of our study are available from Gen Bank under accession numbers: KJ781217.1, KJ781218.1, KM047671, KM013707-KM013712 (Table 2). Molecular analysis of our isolates based on the full-length HA sequences, revealed 97.5% to 98.5%

nucleotide resemblance with the A/California/07/2009 (H1N1) prototype strain. The percentage of nucleotide identity within the HA genes of our isolates was 98% to 99.5.

Table 1. Data of respiratory specimens and sequenced influenza A (H1N1) pdm09 collected from February 2013 to April 2013, in shiraz, Iran.

Season	Sample Tested	Influenza A (H1N1)pdm09 Positive Sample	Mean Age (years)	Male	Female	Sequenced Strains
2013	200	77	10	42	35	9

In all our study strains, S185T mutation located at the Sb antigenic site and S203T mutation situated within the Ca1 antigenic site occurred. S203T mutation is characteristic of clade 7, therefore, all our strains belonged to clade 7. Similarly, the strains isolated in Shiraz during 2010 had S203T mutation; therefore this strains were also included in clade 7.

Table 2. Clinical data and Gen Bank accession numbers for the HA1 subunit (segment 4) of Shiraz influenza A (H1N1) pdm09 circulating strains from February 2013 to April 2013.

Influenza A(H1N1) pdm09 Stains Name	Origin	Date of Collection	Gen Bank Accession	Age (years)	Sex	Clinical Severity
A/shiraz/1/2013	Shiraz	16-Feb-2013	KM013707	26	F	Mild
A/shiraz/5/2013	Shiraz	16-Feb-2013	KM013708	5	M	Mild
A/shiraz/34/2013	Shiraz	18-Feb-2013	KM013709	1	M	Mild
A/shiraz/38/2013	Shiraz	18-Feb-2013	KJ781217	3	F	Mild
A/shiraz/43/2013	Shiraz	10-Mar-2013	KJ781218	16	F	Mild
A/shiraz/87/2013	Shiraz	21-Mar-2013	KM013710	6	F	Mild
A/shiraz/93/2013	Shiraz	21-Mar-2013	KM013711	3	M	Mild
A/shiraz/104/2013	Shiraz	11-Apr-2013	KM013712	7	F	Mild
A/shiraz/125/2013	Shiraz	13-Apr-2013	KM047671	26	M	Moderate

The most common mutations at the antigenic sites of our isolates with respect to vaccine strain (A/California/07/2009) were H138R (9/9) and S203T (9/9) in antigenic site Ca, S185T (9/9) in antigenic site Sb. Among them, H138R and S185T mutations were not observed in strains isolated in Shiraz in 2010. Our study strains indicated other changes at antigenic sites including G140E and E235Q in the Ca antigenic site, Q223R in the vicinity of the Ca antigenic site, K163Q in the Sa antigenic site, Q188H in the Sb antigenic site and A197T in the vicinity of Sb antigenic site. The rest of changes are shown in Table 3.

Table 3. Amino acid substitutions at the HA amino acid sequence of Shiraz influenza A (H1N1) 2009 strains.

Influenza A(H1N1)pdm09 strains	47	83	87	97	119	197	223	234	249	256	262	268	269	283	286	288	297	303	321	374	411	444
A/California/07/2009	V	P	N	D	K	A	Q	V	V	A	G	S	D	K	I	T	P	C	I	E	V	N
A/Shiraz/1/2010	-	S	K	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	V	-	-	-
A/Shiraz/2/2010	-	S	K	-	-	-	R	I	-	-	-	-	-	-	-	-	-	-	V	-	-	-
A/Shiraz/3/2010	-	S	E	-	-	-	R	-	-	-	-	-	-	-	-	-	-	-	V	-	-	-
A/Shiraz/4/2010	-	S	K	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	V	K	-	-
A/Shiraz/5/2010	-	S	K	-		-	-	-	-	-	-	-	-	-	-	-	-	-	-	K	-	-
A/Shiraz/6/2010	-	S	K	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	V	-	-	-
A/Shiraz/7/2010	-	S	K	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A/Shiraz/8/2010	-	S	K	-	-	-	R	-	-	-	-	-	-	-	-	-	-	-	V	K	-	-
A/Shiraz/1/2013	-	S		N	N	T		-	L	-	-	P	E	Q	-	P	L	Y	V	K	-	D
A/Shiraz/5/2013	-	S		N	-	T	-	-	L	T	A	P	-	Q	-	P	L	Y	V	K	-	-
A/Shiraz/34/2013	-	S		N	-	-	-	I	L	-	-	P	E	Q	-	P	L	Y	V	K	-	-
A/Shiraz/38/2013	-	S		N	-	-	-	-	L	-	-	P	-	Q	-	P	L	Y	V	K	-	D
A/Shiraz/43/2013	-	S	K	-	-	-	R	I	L	-	-	P	-	E	-	P	-	Y	V	K	L	D
A/Shiraz/87/2013	-	S		N	-	T	H	-	L	-	-	P	-	Q	-	P	L	Y	V	K	-	D
A/Shiraz/93/2013	-	S	K	-	-	T	-	-	L	-	-	P	-	Q	V	-	L	Y	V	K	-	D
A/Shiraz/104/2013	-	S	K	N	-	-	R	-	L	T	-	P	E	Q	-	P	L	Y	V	K	-	D
A/Shiraz/125/2013	I	S		N	-	-	-	I	L	-	-	P	-	E	-	-	-	Y	V	K	L	D

Table 3. Amino acid substitutions at the HA amino acid sequence of Shiraz influenza A (H1N1) 2009 strains. (Continued)

Influenza A(H1N1)pdm09 strains								*Antigenic sites*										
								Ca	*Ca*	*Sa*	*Sa*	*Sa*	*Sb*	*Sb*	*Ca*	*Ca*	*Ca*	*Ca*
	451	452	455	469	472	499	532	*138*	*140*	*159*	*163*	*164*	*185*	*188*	*203*	*205*	*222*	*235*
A/California/07/2009	S	Q	N	H	D	E	A	H	G	P	K	S	S	Q	S	R	D	E
A/Shiraz/1/2010	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	Q
A/Shiraz/2/2010	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-
A/Shiraz/3/2010	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-
A/Shiraz/4/2010	-	-	-	-	-	-	-	-	-	S	-	T	-	-	T	-	-	-
A/Shiraz/5/2010	-	-	-	-	-	-	-	-	A	-	-	-	-	-	T	-	-	-
A/Shiraz/6/2010	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	K	-	-
A/Shiraz/7/2010	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	G	-
A/Shiraz/8/2010	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-
A/Shiraz/1/2013	N	-	-	-	N	K	E	R	-	-	Q	-	T	H	T	-	-	-
A/Shiraz/5/2013	N	R	K	-	N	K	E	R	-	-	-	-	T	-	T	-	-	-
A/Shiraz/34/2013	N	-	K	-	N	K	E	R	-	-	-	-	T	H	T	-	-	-
A/Shiraz/38/2013	N	R	K	-	N	K	E	R	-	-	-	-	T	-	T	-	-	-
A/Shiraz/43/2013	N	-	K	-	-	K	E	R	E	-	-	-	T	H	T	-	-	Q
A/Shiraz/87/2013	N	-	K	-	-	K	E	R	-	-	-	-	T	-	T	-	-	-
A/Shiraz/93/2013	N	-	K	D	N	K	E	R	-	-	-	-	T	-	T	-	-	-
A/Shiraz/104/2013	N	-	K	-	N	K	E	R	-	-	-	-	T	-	T	-	-	-
A/Shiraz/125/2013	N	-	-	-	-	K	E	R	-	-	-	-	T	-	T	-	-	-

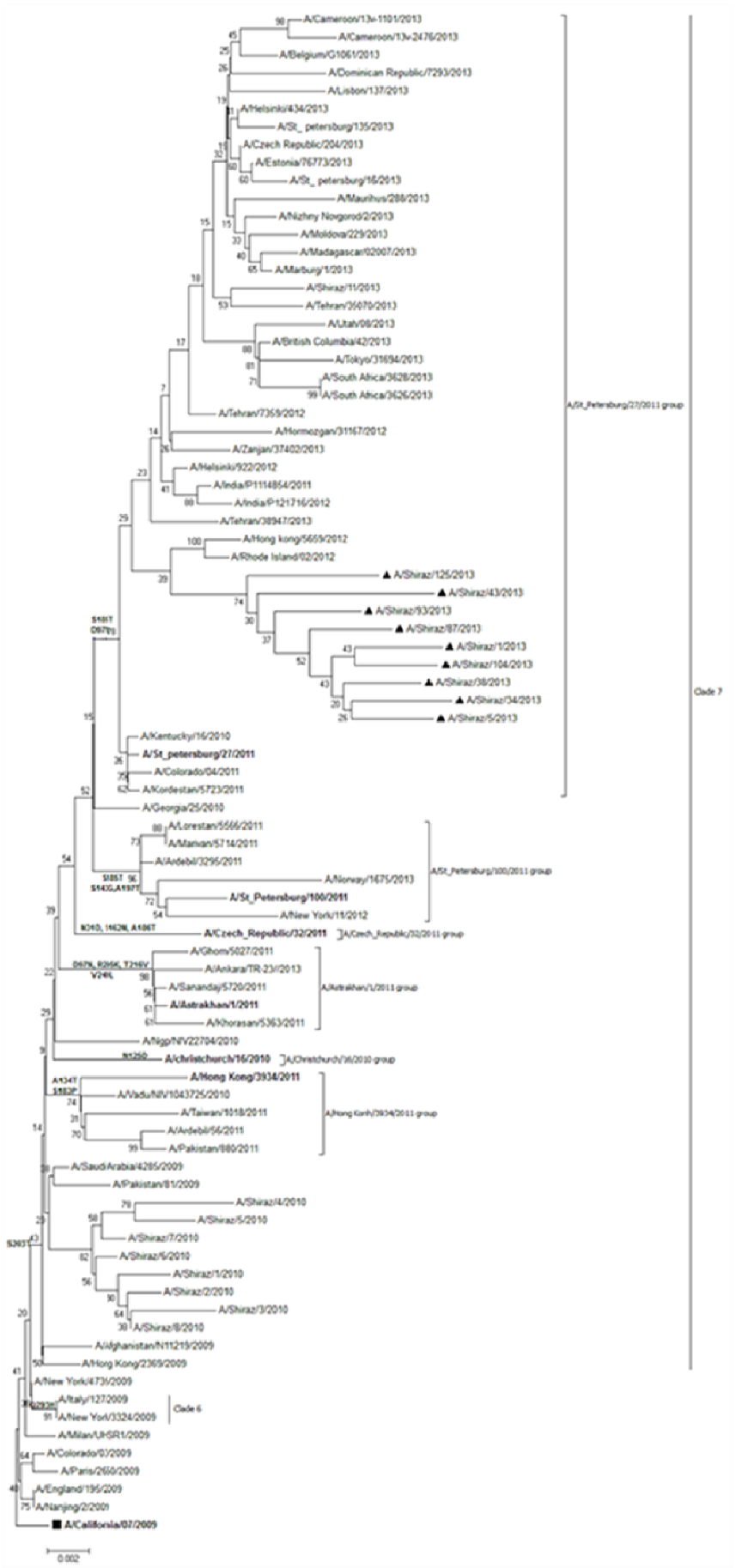

Figure 1. Phylogenetic tree for haemagglutinin (HA) genes of influenza A (H1N1) pdm09 viruses in Shiraz. The tree was rooted with the vaccine strain A/California/07/2009 as the outgroup. The tree was constructed using the neighbor-joining method with Kimura two-parameter distances using MEGA 6 software.

Our study strains, like other A (H1N1) pdm09 viruses, had the E374K mutation, located in a site involved in membrane fusion (21,22). In addition, among our study strains, there is one strain that has T288P mutation that not only leading to loss of a glycosylation site and finally mild disease outcome, also increases the possibility of immune escape.

Phylogenetic analysis of the HA sequences revealed that 2010 and 2013 strains did not form a shared monophyletic group, suggesting that they originate from different introductions to human population and our study strains clustered with more recent strains, especially strains from 2013 than from 2009-2012.

DISCUSSION

This study was carried out to analyze the molecular diversity of pandemic influenza A (H1N1) viruses in Shiraz during February-April 2013.

Since emergence of influenza A (H1N1) pdm09 in Mexico in 2009, several studies have been performed in different countries to determine gene variations of pandemic strains of influenza A (H1N1) pdm09 (11,23), especially HA genes, which contribute to viral attachment and immunization (11,24,25).

A total of 77 sampled patients (38.5%) had confirmed pandemic influenza A (H1N1) virus infection in Shiraz during February 2013 to April 2013. Most patients belonged to young and child population with a mean age of 10 years. In agreement, CDC reported that individuals aged 5-14 years have the highest infection frequency. High level antibody in elder population due to previous contact with this type of virus (such as 1918 pandemic flu), may explain this finding. In contrast, younger individuals, because of having a low or negative antibody, are more susceptible to pandemic flu (15). In addition, high prevalence of influenza in children may be due to high transmission in the school environment (14). This finding revealed the necessity of vaccination of younger people and children (15).

Vaccination is the principal way for preventing influenza infection and decreasing its impact (26). Comparison of our sequenced isolates to the vaccine virus A/California/07/2009 gives useful information for evaluating the efficacy of the vaccine. The sequencing studies of the HA genes showed 97.5%-98.5% nucleotide identity with respect to prototype vaccine strain A/California/07/2009. Therefore, all of our A (H1N1) pdm09 strains were the genetic variants of the reference vaccine strain (A/California/07/2009). In comparison, strains in Shiraz in 2010 revealed 98.6%-99.3% nucleotide similarity to vaccine strain A/California/07/2009 and 98.5%-99.7% nucleotide identity between each other.

Genetic variations in Shiraz virus strains in 2013 were more than the isolates in 2010 that may be due to the accumulation of mutations in recent years.

Analysis of amino acid sequences of HA gene (from aa 1 to 566) indicated mutations in antigenic sites situated in HA1 domain, including Sa, Sb, Ca, and Cb (21) which are responsible for immunization and viral attachment (24,25,27). In addition, some mutations were seen in the HA2 region.

Interestingly, all of the strains isolated in 2010 showed proline at 83 amino acid position whereas all of 2013 strains were similar to vaccine strains at this position. It may be supposed that this mutation was not useful for survival of the virus, so it is deleted under selective pressure.

As mentioned before, our study strains revealed substitutions at or in the vicinity of the antigenic sites. Most of these changes have been reported in viruses from different countries which are available in Gen Bank. S185T and S203T mutations almost have been observed in all of the influenza pandemic strains for example in India (2011-2013), Thailand (2010-2011), Taiwan (2011, 2013) and United States (2009-2013). H138R and Q223R mutations were just reported in Asia. H138R was reported in Beijing-China in 2013 (28) and Q223R (H) was observed in Korea (2009) and Iran (2010, 2012). Mutation in position 163 has been reported in Mexico in 2012 (K163R) (21). But Q188H mutation was only observed in our study strains and E235Q substitution was observed only in one strain isolated in Shiraz-Iran in 2010. T288P mutation was also reported in Greece in 2013 and this change was seen in patients with mild illness (23). Although a few samples were studied, the same amino acid substitutions in all the analyzed viruses indicated that these changes are common characteristics of the strains circulating in Shiraz in 2013.

D222G mutation was seen in one of the circulating Shiraz virus strain in 2010 in a fatal case (available in Gen Bank), but in our study strains, this mutation was not observed.

Amino acid position 222 situated in Ca antigenic site and it is a part of receptor binding site therefore, change in this site may alter the receptor binding function and influence severity of the disease. D222G mutation was the main change related to severe or fatal disease of H1N1 (pdm09) virus. Nevertheless, the pandemic (H1N1) 2009 viruses with 222G mutation have been antigenically close to the vaccine strain A/California/7/2009(29). Similar to the majority of (H1N1)pdm09 viruses, the eight putative glycosylation sites are conserved in HA gene at positions27, 28, 40, 104, 293, 304, 498 and 557 (30). But change at position 304(P304S)was detected in one of the strains in Shiraz in 2010, with a possible impact on the glycosylation and consequent alteration in antigenicity and pathogenicity (31)

Nelson *et al.* (2009) reported A (H1N1) pdm09 strains formed 7 phylogenetically distinct clades which disseminated all over the world (24). All our sequenced isolates belonged to clade 7 of A (H1N1) pdm09 virus having S203T mutation, which is the signature substitution of clade7. This position is located in sb antigenic site of the HA1 region and may play an important role in antigenic drift. The same mutation was observed in strains of Shiraz in 2010, so we also included 2010 strains in clade 7. Our data revealed that the predominant H1N1 pdm09 virus in Shiraz was clade 7; this was in agreement with previous reports which clade 7 viruses constitute the dominant circulating strains in all of the world (7).

Based on the phylogenetic analysis, the HA genes are grouped into eight different genetic groups (31). Phylogenetic tree, was built based on HA gene sequences of our strains and 83 other global strains rooted with reference strains of A (H1N1) pdm09, showed effectively all our strains are related to each other and clustered with clade 7 global reference. Among our 9 study strains seven strains fell into genetic group 6. These strains were characterized by S185T and S203T mutation at the antigenic site Sb, and D97N mutation. Although two isolates do not have the D97N mutation, in phylogenetic tree they are clustered into the rest of our strains and we may consider them in genetic group 6. It is concluded that strains circulating in this locality had probably one origin and our stem is probably a cluster that has evolved from a common ancestor.

In the WHO report pandemic (H1N1) 2009-update 78, it was mentioned that all of the pandemic influenza A (H1N1) viruses worldwide were closely related genetically and

antigenically to the vaccine strains (11). In agreement with WHO report, the sequence of HA genes of the 9 pandemic A (H1N1) virus isolates in Shiraz and vaccine strain showed high homology to each other. Although variation at or in the vicinity of the antigenic sites may be antigenically significant and affect the vaccine efficacy (30), such an influence on the antigenecity has not been reported so far even for the known mutations (12,21,28). But the exact impact of these mutations, especially Q188H mutation, on the antigenicity, are still unknown and require more studies with a larger sample size

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