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Analysis of the Hemagglutinin and Neuraminidase Genes of Human Influenza A/H3N2 Viruses Circulating in Iran between 2005 and 2007: Antigenic and Phylogenetic Relationships to Vaccine Strains

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Key Words

Influenza A/H3N2 virus · Iranian isolates · Antigenic variations · Surface glycoproteins

Abstract

Objectives: To study the antigenic variations in influenza A/H3N2 viruses circulating in Iran for characterization and phylogenetic relationships to vaccine strains. ***Methods:*** RT-PCR, full sequencing of hemagglutinin (HA) and neuraminidase (NA) genes and analysis by sequence handling and phylogenetic programs were done. ***Results:*** The HA sequences of 2007 isolates fell within the clade represented by the HA of A/Brisbane/10/07 and characterized by the amino acid changes relative to the HA of A/Wisconsin/67/05, G50E and K140I. The only isolate in 2006 fell within A/Berlin/02/06 with V112I and K173E changes. The 2005 isolates characterized by Y159F, S189N and S227P changes within A/California/07/04. In all isolates we had E190D which is important because this was responsible for the loss of ability of A/H3N2 viruses to bind to chicken red blood cells. There were some substitutions in the antigenic sites of the HA. Similar to other studies, conserved residues for catalytic sites and also framework sites of NA supporting the catalytic residues were detected. We had some changes in the variable regions of the NA head domain. ***Conclusion:*** Comparison between Iranian viruses and vaccine strains showed high similarity between them and vaccine strains used in the northern hemisphere.

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Introduction

Influenza A viruses are negative-stranded RNA viruses belong to the *Orthomyxoviridae* family [1]. They have two surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA). 16 distinct subtypes of HA and 9 subtypes of NA have been recognized so far. Based on the antigenic properties of these two glycoproteins, influenza viruses are classified into different subtypes [2].

The HA glycoprotein is constructed of two domains: globular head (composed of most of the HA1 polypeptide of HA) and long fibrous stem (comprised mostly of the HA2 polypeptide). The HA1 segment of the influenza HA protein is the most rapidly evolving region of the virus and plays a major role in viral attachment and evasion from neutralizing antibody responses [3]. On the globular head of A/H3N2 viruses five antigenic sites (A–E) have been identified [3–5] where antibody binding can occur [6, 7]. 18 codons in the HA1 segment of the HA

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Table 1. List of primers used for RT-PCR and sequencing

Gene name	Primer	Sequence 5′–3′
H3		
HA1	H3HAF6*	AAGCAGGGGATAATTCTATTAACC
	H3HAF567	CTGAACGTGACTATGCCAAACAAT
	H3AR650	TTGGTCACTGTCCGTACTCGGGTG
	H3HAR1075*	AACCGTACCAACCRTCCACCATTC
HA2	H3HAF567*	CTGAACGTGACTATGCCAAACAAT
	H3A1F1	ATGAAGACTATCATTGCTTTCAGC
	H3A2F1	AGGCATATTCGGCGCAATCGCAGG
	H3HAR1075	AACCGTACCAACCRTCCACCATTC
	H3A1R1*	GTCTATCATTCCCTCCCAACCATT
N2		
NA-5′[1]	N2F1*	AGCAAAAGCAGGAGTGAAAATGAA
	N2F387	CATGCGATCCTGACAAGTGTTATC
	N2F754	TGCTTCAGGAAAAGCTGATACTAA
	N2R410	GATAACACTTGCTAGGATCGCATG
	N2R778	TTAGTATCAGCTTTTTCTGAAGCA
	N2R1104*	ATCCACACGTCATTTCCATCGTCA
NA-3′[1]	N2F387*	CATGCGATCCTGACAAGTGTTATC
	N2F754	TGCTTCAGGAAAAGCTGATACTAA
	N2R778	TTAGTATCAGCTTTTTCTGAAGCA
	N2R1104	ATCCACACGTCATTTCCATCGTCA
	N2R1*	TTCTAAAATTGCGAAAGCTTATAT

* These are the primers used in RT-PCR.
[1] NA-5′ and NA-3′ are two overlapping fragments to amplify the whole neuraminidase gene; these are located at the 5′ and 3′ ends of the genomic sequence.

genes of human influenza A/H3N2 appear to be under positive selection to change the amino acids they encode [8–11].

NA is the second major glycoprotein of influenza A viruses. NA is a tetramer comprised of a bulky head attached to a slender stalk [12]. Viral NA removes terminal sialic acids from glycoconjugates on both cell receptors and the viral HA, thereby allowing the release of progeny viruses from infected cells [13–15].

The influenza virus glycoproteins are well known to undergo antigenic drift and shift. Antigenic drift occurs because of the point mutations result in amino acid changes in the two surface glycoproteins and enables viruses to escape from preexisting immunity and emergence of new antigenic variants. These new variants are the cause of the worldwide seasonal influenza epidemics. Because of the high morbidity and mortality due to influenza epidemics, monitoring the antigenic variations in circulating influenza viruses is crucial for anticipating epidemics and vaccine design [3, 4, 16].

Antigenic shift is a major change that results in the emergence of a new subtype and usually causes a pandemic, such as the three pandemics of the 20th century and the currently emerging pandemic of the 21st century [6].

The variability in both the emerging pandemic and seasonal influenza viruses is a considerable problem for the production of an effective vaccine and the reassessment of vaccine composition according to the circulating strains.

In this study sequencing analysis of the HA and NA of influenza A/H3N2 was performed on viruses from the Islamic Republic of Iran, in order to describe the virus variation and mutations which differ from the annually recommended vaccine strains in this region of the world.

Materials and Methods

Clinical Samples

Clinical samples were collected from 432 individuals who where diagnosed with influenza-like illness between January 2005 and October 2008. No A/H3N2 viruses were isolated during 2008 which may have been because A/H1N1 viruses predominated in Iran during that season.

The specimens were provided by Iran Ministry of Health and sent to the National Influenza Centre at Tehran University of Medical Sciences.

Virus Screening and Isolation

In order to screen the influenza virus A/H3N2, RNA extraction and real-time PCR were done on samples and the positive ones were inoculated into MDCK cells for virus isolation and culture.

Genetic and Phylogenetic Analysis

All positive samples were subjected to HA and NA gene sequencing at the WHO Influenza Centre, National Institute for Medical Research, London. RNA was extracted from 140 μl of cell culture supernatants (in case of positive cell culture) or from 140 μl of clinical samples using the Qiagen viral RNA Mini Kit. The full HA and NA genes were amplified using the primers shown in table 1. RT-PCR conditions with the Qiagen One Step RT-PCR Kit were as follows: cDNA synthesis (60° for 1 min, 50° for 30 min and 95° for 15 min), followed by 40 amplification cycles consisting of denaturation at 94° for 30 s, primer annealing at 50° for 30 s and extension at 72° for 1 min with a final extension at 72° for 10 min. The expected size of the PCR products were analyzed by electrophoresis using 1% agarose gels.

The PCR products were purified using GFX PCR DNA and Gel Bond Purification Kit (GE Healthcare). DNA sequencing was performed using the BigDye Terminator V1.1 Cycle Sequencing Kit. After ethanol precipitation the reactions were resolved on the Mega BACE sequencing machine and the HA and NA sequences were edited with the Staden package (http://www.sanger.ac.uk/Software/production/staden/) and aligned using the Wisconsin package (http://accelrys.com/products/gcg_wisconsin_package or http//www.gcg.com). Phylogenetic trees were constructed by means of the Neighbor-Joining method using the Wisconsin

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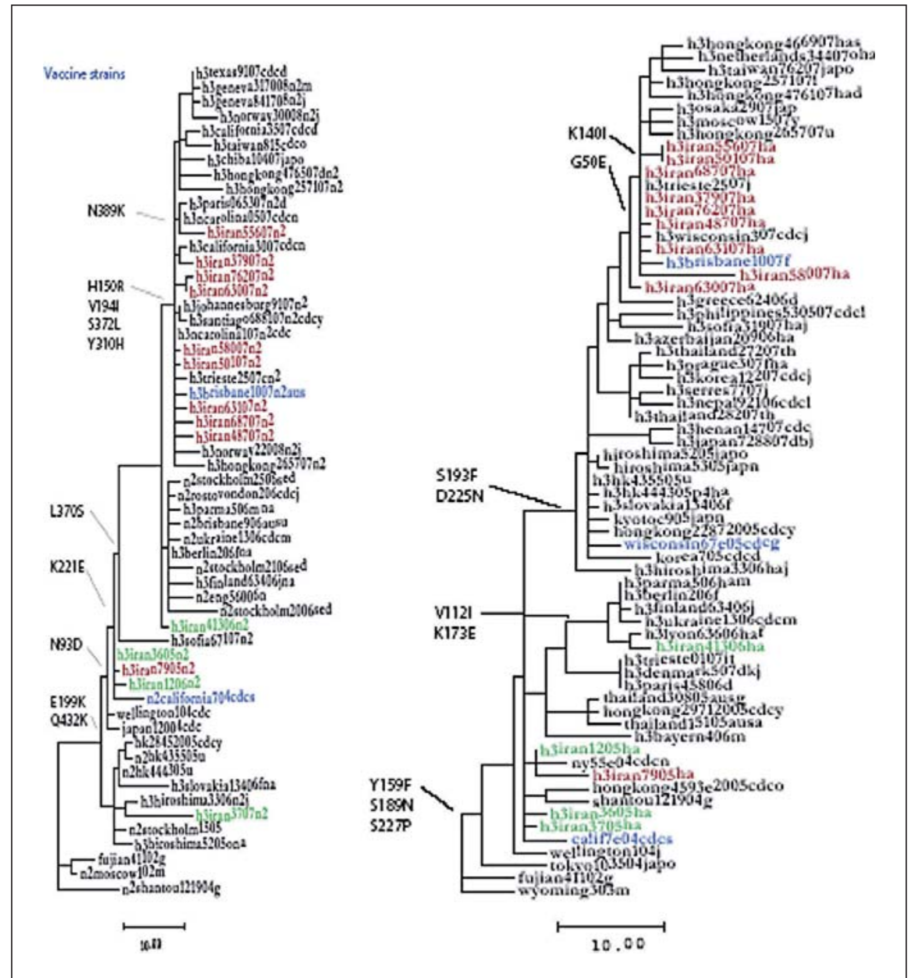

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Fig. 1. Phylogram of HA and NA genes of Iranian H3N2 viruses.

package. The lengths of the horizontal lines are proportional to the numbers of nucleotide differences, as indicated by the bar (fig. 1). All sequences determined by this study have been deposited in the GenBank database under accession numbers FJ769866–FJ769915.

Results

14 A/H3N2 subtypes were found in all throat swab specimens from patients with influenza-like illness between 2005 and 2008. We failed to isolate the A/H3N2 subtype during this study in 2008. The full genome of HA and NA genes were sequenced and analyzed. The deduced amino acid comparison between our viruses and vaccine strains showed high similarity between them and reference strains and vaccine strains which were used in the northern hemisphere between 2005 and 2007.

Phylogenetic analysis for HA and NA of A/H3N2 isolates showed continuous evolution and similarity to the vaccine strains of each 3 years. Beside the vaccine strains, Iranian A/H3N2 viruses were almost exclusively similar to the strains which were circulating during each of those years all over the world.

Five altered amino acids were detected at the antigenic sites A, B, C and E of HA1 with different changes in three consecutive years (table 2).

11 Glycosylation sequons [17] which are N-linked glycosylation sites composed of Asn-X-Ser/Thr were found to be similar to the other studies: in this sequence X can be any amino acid except for aspartic acid and proline. In some strains there were mutations which have changed N-linked glycosylation sites, for instance A/Tehran/79/05 had N8T and A/Tehran/487/07 had N63K (fig. 2), both resulting in the loss of the glycosylation sequon. All isolates had an asparagine residue at position 145 (K145N),

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Table 2. Amino acid substitutions in the hemagglutinin gene of influenza virus A/H3N2 isolated in Iran

Isolate name	Date of collection	Amino acid position of HA gene																			
		50(C)	112	140(A)	173(E)	193(B)	199(B)	225	375	121*	8	63	145(A)	159(B)	172	189(B)	190(D)	209	226(D)	227	278
A/Iran/12/05	25/2/2005	G	V	K	K	S	S	D	N	R	N	N	N	F	Q	N	D	S	I	P	N
A/Iran/36/05	12/2/2005	G	V	K	K	S	S	D	N	R	N	N	N	F	Q	N	D	S	I	P	N
A/Iran/37/05	13/2/2005	G	V	K	K	S	S	D	N	R	N	N	N	F	Q	N	D	S	I	P	N
A/Iran/79/05	4/1/2005	G	V	K	K	S	S	D	N	R	T	N	N	F	Q	N	D	S	I	P	N
A/Iran/413/06	15/2/2006	G	I	K	E	S	P	D	D	R	N	N	N	F	R	N	D	S	I	P	K
A/Iran/580/07	27/1/2007	E	V	I	K	F	S	N	D	K	N	N	N	F	Q	N	D	S	I	P	N
A/Iran/487/07	21/1/2007	E	V	I	K	F	S	N	D	K	N	K	N	F	Q	N	D	S	I	P	N
A/Iran/631/07	2/2/2007	E	V	I	K	F	S	N	D	K	N	N	N	F	Q	N	D	S	I	P	N
A/Iran/687/07	6/2/2007	E	V	I	K	F	S	N	D	K	N	N	N	F	Q	N	D	S	I	P	N
A/Iran/379/07	12/1/2007	E	V	I	K	F	S	N	D	K	N	N	N	F	Q	N	D	S	I	P	N
A/Iran/762/07	11/2/2007	E	V	I	K	F	S	N	D	K	N	N	N	F	Q	N	D	S	I	P	N
A/Iran/501/07	19/1/2007	E	V	I	K	F	S	N	D	K	N	N	N	F	Q	N	D	T	I	P	N
A/Iran/556/07	26/2/2007	E	V	I	K	F	S	N	D	K	N	N	N	F	Q	N	D	T	I	P	N
A/Iran/630/07	2/2/2007	E	V	I	K	F	S	N	D	K	N	N	N	F	Q	N	D	S	I	P	N

A, B, C and E are the antigenic sites. * HA2 numbering.

which created a glycosylation site in antigenic site A, similar to the previous studies [6, 18–20]. K145N substitution is characteristic of viruses similar to A/California/7/2004. This change is an exchange of a positive to a polar amino acid and there is some evidence that this might be responsible for an antigenic drift at that time [20, 21].

In all isolates E190D was detected which is important because some experiments revealed that this change was responsible for the loss of ability of A/H3N2 viruses to bind to chicken red blood cells [22]. In 2005 and 2006 isolates there were asparagine in position 193 (F193S): 193 is located in antigenic site B and is able to influence the antigenicity of the HA [19].

Some substitutions similar to the previous reports were Y159F, S189N in antigenic site B and V226I, S227P in antigenic site D. Ile-226, Pro-227 and Ser-228 are responsible for the NeuAcα2,6 Gal linkage which is very important in receptor binding [21, 23, 24].

One substitution in HA1, S209T in antigenic site D, was observed in two isolates of 2007 (table 2).

Two mutations, i.e. N278K which is typical of Lyon/636 and Q172R (HA2 numbering), were found in the one isolate detected in 2006. It fell within the phylogenetic group characterized by amino acid changes V112I and K173E and represented by A/Berlin/2/2006 and A/Lyon/636/2006. Some other changes have been found which are shown in table 2. These changes were mostly at the antigenic sites and they were important in the antigenic drift. HA phylogeny showed the HA sequences were at the same branch of vaccine strains and there was no evidence of a significant distinct subclade of viruses circulating in Iran over the period of study (fig. 1).

In comparison of nucleotide and deducted amino acid sequences of NA from these viruses, a high degree of similarity was found between these isolates and vaccine strains in each year. Similar to the other studies [6, 25] conserved residues for catalytic sites (R118, D151, R152, R224, E276, R292, R371 and Y406) and also framework sites supporting the catalytic residues (E119, R156, W178, D198, I222, E227, H274, E277, N294 and E425) were detected. We found some different changes in NA which are shown in table 3.

The variable regions in the NA head domain are amino acids 140–155, 328–370 and to a lesser extent 381–403 [26, 27] and we also found some changes at these locations (table 3). The NA sequences of these viruses fell into the corresponding clade and were similar to those of equivalent viruses circulating in those 3 years and there was no evidence for the emergence of a significant distinct subclade (fig. 1).

Fig. 2. Amino acid comparison between HA protein of Iranian H3N2 viruses and vaccine strains. Lines represent amino acids similar to the consensus. The N-linked glycosylation sites are shown in grey (red in the online version).

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```
                                1                                                       50
h3iran63007ha      ____ __________ __________ __________ __________ _____e____ __________ __________
h3iran55607ha      ____ __________ __________ __________ __________ _____e____ __________ __________
h3iran50107ha      ____ __________ __________ __________ __________ _____e____ __________ __________
h3iran76207ha      ____ __________ __________ __________ __________ _____e____ __________ __________
h3iran37907ha      ____ __________ __________ __________ __________ _____e____ __________ __________
h3iran68707ha      ____ __________ __________ __________ __________ _____e____ __________ __________
h3iran63107ha      ____ __________ __________ __________ __________ _____e____ __________ __________
h3iran48707ha      ____ __________ __________ __________ __________ _____e____ ________K_ __________
h3iran58007ha      ____ __________ __________ __________ __________ _____e____ __________ __________
h3brisbane1007F    ____ __________ __________ __________ __________ _____e____ __________ __________
h3iran41306ha      ____ __________ __________ __________ __________ __________ __________ __________
h3berlin206F       ____ __________ __________ __________ __________ __________ __________ __________
h3iran3605ha       ____ __________ __________ __________ __________ __________ __________ __________
h3iran1205ha       ____ __________ __________ __________ __________ __________ __________ __________
h3iran3705ha       ____ __________ __________ __________ __________ __________ __________ __________
h3iran7905ha       ____ ___T______ __________ __________ __________ __________ __________ __________
calif7e04cdcS      ____ __________ __________ __________ __________ __________ __________ __________

Consensus          QKLP GNDNSTATLC LGHHAVPNGT IVKTITNDQI EVTNATELVQ SSSTGGICDS PHQILDGENC TLIDALLGDP

                                                                      112                   140        173
h3iran63007ha      __________ __________ __________ __________ __________ __________ __________ __________ __________ __________
h3iran55607ha      __________ __________ __________ __________ __________ __________ _____i____ __________ __________ __________
h3iran50107ha      __________ __________ __________ __________ __________ __________ _____i____ __________ __________ __________
h3iran76207ha      __________ __________ __________ __________ __________ __________ _____i____ __________ __________ __________
h3iran37907ha      __________ __________ __________ __________ __________ __________ _____i____ __________ __________ __________
h3iran68707ha      __________ __________ __________ __________ __________ __________ _____i____ __________ __________ __________
h3iran63107ha      __________ __________ __________ __________ __________ __________ _____i____ __________ __________ __________
h3iran48707ha      __________ __________ __________ __________ __________ __________ _____i____ __________ __________ __________
h3iran58007ha      __________ __________ __________ __________ __________ __________ _____i____ __________ __________ __________
h3brisbane1007F    __________ __________ __________ __________ __________ __________ _____i____ __________ __________ __________
h3iran41306ha      __________ __________ __________ __________ __________ __________ __________ __________ __________ ________e_
h3berlin206F       __________ __________ __________ _______i__ __________ __________ __________ __________ __________ ________e_
h3iran3605ha       __________ __________ __________ __________ __________ __________ __________ __________ __________ __________
h3iran1205ha       __________ __________ __________ __________ __________ __________ __________ __________ __________ __________
h3iran3705ha       __________ __________ __________ __________ __________ __________ __________ __________ __________ __________
h3iran7905ha       __________ __________ __________ __________ __________ __________ __________ __________ __________ __________
calif7e04cdcS      __________ __________ __________ __________ __________ __________ ___s______ __________ __________ __________

Consensus          QCDGFQNKKW DLFVERSKAY SNCYPYDVPD YASLRSLVAS SGTLEFNNES FNWTGVTQNG TSSACKRRSN NSFFSRLNWL THLKFKYPAL NVTMPNNEKF

                                                          193   199                          225
h3iran63007ha      __________ __________ __________ __________ __________ __________ __________ __________ __________ __________
h3iran55607ha      __________ __________ __________ ____t_____ __________ __________ __________ __________ __________ __________
h3iran50107ha      __________ __________ __________ ____t_____ __________ __________ __________ __________ __________ __________
h3iran76207ha      __________ __________ __________ __________ __________ __________ __________ __________ __________ __________
h3iran37907ha      __________ __________ __________ __________ __________ __________ __________ __________ __________ __________
h3iran68707ha      __________ __________ __________ __________ __________ __________ __________ __________ __________ __________
h3iran63107ha      __________ __________ __________ __________ __________ __________ __________ __________ __________ __________
h3iran48707ha      __________ __________ __________ __________ __________ __________ __________ __________ __________ __________
h3iran58007ha      __________ __________ __________ __________ __________ __________ __________ __________ __________ __________
h3brisbane1007F    __________ __________ __________ __________ __________ __________ __________ __________ __________ __________
h3iran41306ha      __________ ________s_ ____p_____ __________ __________ d_________ __________ __________ __________ __________
h3berlin206F       __________ ________s_ ____p_____ __________ __________ d_________ __________ __________ __________ __________
h3iran3605ha       __________ ________s_ __________ __________ __________ d_________ __________ __________ __________ __________
h3iran1205ha       __________ ________s_ __________ __________ __________ d_________ __________ __________ __________ __________
h3iran3705ha       __________ ________s_ __________ __________ __________ d_________ __________ __________ __________ __________
h3iran7905ha       __________ ________s_ __________ __________ __________ d_________ __________ __________ __________ __________
calif7e04cdcS      __________ ___n____s_ _t________ __________ __________ d_________ __________ __________ __________ __________
Consensus          DKLYIWGVHH PGTDNDQIFL YAQASGRITV STKRSQQTVI PNIGSRPRVR NIPSRISIYW TIVKPGDILL INSTGNLIAP RGYFKIRSGK SSIMRSDAPI

h3iran63007ha      __________ __________ __________ __________ __________ __________ __________ __________ __________ __________
h3iran55607ha      __________ __________ __________ __________ __________ __________ __________ __________ __________ __________
h3iran50107ha      __________ __________ __________ __________ __________ __________ __________ __________ __________ __________
h3iran76207ha      __________ __________ __________ __________ __________ __________ __________ __________ __________ __________
h3iran37907ha      __________ __________ __________ __________ __________ __________ __________ __________ __________ __________
h3iran68707ha      __________ __________ __________ __________ __________ __________ __________ __________ __________ __________
h3iran63107ha      __________ __________ __________ __________ __________ __________ __________ __________ __________ __________
h3iran48707ha      __________ __________ __________ __________ __________ __________ __________ __________ __________ __________
h3iran58007ha      __________ __________ __________ __________ __________ __________ __________ __________ __________ __________
h3brisbane1007F    __________ __________ __________ __________ __________ __________ __________ __________ __________ __________
h3iran41306ha      ___K______ __________ __________ __________ __________ __________ __________ __________ __________ __________
h3berlin206F       __________ __________ __________ __________ _________~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
h3iran3605ha       __________ __________ __________ __________ __________ __________ __________ __________ __________ __________
h3iran1205ha       __________ __________ __________ __________ __________ __________ __________ __________ __________ __________
h3iran3705ha       __________ __________ __________ __________ __________ __________ __________ __________ __________ __________
h3iran7905ha       __________ __________ __________ __________ __________ __________ __________ __________ __________ __________
calif7e04cdcS      __________ __________ __________ __________ __________ __________ __________ __________ __________ __________

Consensus          GKCNSECITP NGSIPNDKPF QNVNRITYGA CPRYVKQNTL KLATGMRNVP EKQTRGIFGA IAGFIENGWE GMVDGWYGFR HQNSEGIGQA ADLKSTQAAI

                   375                                                                         450
h3iran63007ha      __________ __________ __________ __________ __________ __________ __________ __________ __________ __________
h3iran55607ha      __________ __________ __________ __________ __________ __________ __________ __________ __________ __________
h3iran50107ha      __________ __________ __________ __________ __________ __________ __________ __________ __________ __________
h3iran76207ha      __________ __________ __________ __________ __________ __________ __________ __________ __________ __________
h3iran37907ha      __________ __________ __________ __________ __________ __________ __________ __________ __________ __________
h3iran68707ha      __________ __________ __________ __________ __________ __________ __________ __________ __________ __________
h3iran63107ha      __________ __________ __________ __________ __________ __________ __________ __________ __________ __________
h3iran48707ha      __________ __________ __d_______ __________ __________ __________ __________ __________ __________ __________
h3iran58007ha      __________ __________ __________ __________ __________ __________ __________ __________ __________ __________
h3brisbane1007F    __________ __________ __________ __________ __________ __________ __________ __________ __________ __________
h3iran41306ha      __________ __________ __________ __________ __________ __________ __________ _____r____ __________ __________
h3berlin206F       ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
h3iran3607ha       n_________ __________ __________ __________ __________ __________ __________ _____r____ __________ __________
h3iran1207ha       n_________ __________ __________ __________ __________ __________ __________ _____r____ __________ __________
h3iran3707ha       n_________ __________ __________ __________ __________ __________ __________ _____r____ __________ __________
h3iran7907ha       n_________ __________ __________ __________ __________ __________ __________ _____r____ __________ __________
calif7e04cdcS      n_________ __________ __________ __________ __________ __________ __________ _____r____ __________ __________
                   Consensus  DQINGKLNRL IGKTNEKFHQ IEKEFSEVEG RIQDLEKYVE DTKIDLWSYN AELLVALENQ HTIDLTDSEM NKLFERTKKQ GCFKIYHKCD
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Table 3. Amino acid substitutions in the neuraminidase gene of influenza virus A/H3N2 isolated in Iran

Isolate name	Date of collection	Amino acid position of HA gene					
		150	194	310	310	372	387
A/Iran/12/05	25/02/2005	H	V	Y	L	S	N
A/Iran/36/05	12/02/2005	H	V	Y	L	S	N
A/Iran/37/05	13/02/2005	H	V	Y	L	S	N
A/Iran/79/05	04/01/2005	H	V	Y	L	S	N
A/Iran/413/06	15/02/2006	R	I	H	S	L	N
A/Iran/580/07	27/01/2007	R	I	H	S	L	K
A/Iran/487/07	21/01/2007	R	I	H	S	L	K
A/Iran/631/07	02/02/2007	R	I	H	S	L	K
A/Iran/687/07	06/02/2007	R	I	H	S	L	K
A/Iran/379/07	12/01/2007	R	I	H	S	L	K
A/Iran/762/07	11/02/2007	R	I	H	S	L	K
A/Iran/501/07	19/01/2007	R	I	H	S	L	K
A/Iran/556/07	26/02/2007	R	I	H	S	L	K
A/Iran/630/07	02/02/2007	R	I	H	S	L	K

Discussion

From January 2005 to October 2008, 432 specimens were collected of which 50 (11.33%) were influenza B, 35 (8.10%) were A/H1N1, and 14 (3.24%) were A/H3N2 viruses. Generally all over the world there was a variable frequency of A/H1N1, A/H3N2 and influenza B virus circulation in those years, e.g. according to the report by the WHO Influenza Centre in London: of some 800 influenza viruses isolated in 34 countries from February 2005 to 2006, approximately half (49%) were A/H3N2, 17% were A/H1N1 and 34% were influenza B. In 2006–2007 some 780 influenza viruses, isolated in 42 countries, were characterized. These included similar numbers of A/H1N1 (32%), A/H3N2 (31%) and B (37%) viruses. During February to September 2007, influenza A/H1N1, A/H3N2 and B viruses continued to circulate worldwide and caused outbreaks in many countries. Influenza A/H3N2 viruses predominated overall and comprised roughly half of the viruses isolated. From 700 influenza viruses isolated in 30 countries during October 2007 to February 2008, the majority were A/H1N1 (77%) and only a few (2%) were of the A/H3N2 subtype [28].

In the 14 samples of A/H3N2 viruses, the changes seen in HA and NA were compared with vaccine strains. The HA sequences of 2005 isolates were characterized by Y159F, S189N and S227P changes within A/California/07/04. The only isolate in 2006 fell within A/Berlin/02/06 with V112I and K173E changes. The 2007 isolates fell within the clade represented by the HA of A/Brisbane/10/07 and characterized by the amino acid changes relative to the HA of A/Wisconsin/67/05, G50E and K140I. We failed to isolate any A/H3N2 in 2008.

The HA1 segment showed higher changes in than HA2 because of its antigenic sites and receptor-binding properties as generally seen. Changes in antigenic sites are the center of interest because of the need for the continual update of influenza vaccines. The residues within the receptor-binding site are relatively conserved but the residue mainly responsible for NeuAcα 2,6 Gal linkage specific for the H3 subtype, residue 226, was Ile-226 instead of Leu-226 as previously reported [6, 23, 29–32]. An amino acid substitution at position 145 which is located adjacent to antibody-binding site A and within a known glycosylation site results in a more accessible receptor-binding cleft located directly above residue 145 [20, 21]. N-linked glycosylation is important because its presence or absence may cause a loss of function of the glycoprotein and may initiate and maintain folding, stability, transport, antigenicity and immunogenicity of the protein [6]. A virus containing HA with little carbohydrate modification can tightly bind the receptor, requiring greater NA activity to promote particle release. Conversely, an HA with more extensive glycosylation interacts weakly with receptors and requires a less active NA to facilitate release. Overall, the HA depends on a balance of glycosylation to mediate the proper folding of the HA, interaction of virus with receptor, and efficient particle release [33, 34].

In the context of the NA gene, conserved catalytic and framework sites of the enzyme as in previous studies have been found [6], but we, as expected, still found some

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changes in the NA of the viruses and showed a high similarity to vaccine strains in each year.

To date, molecular and phylogenetic analysis of influenza A/H3N2 viruses spanning 3 consecutive years have not been reported in Iran. Comparison of the nucleotide and amino acid sequences of the HA and NA genes between A/H3N2 isolates and the vaccine strains showed they are closely related to the vaccine strains recommended for those 3 years. Phylogenetic analyses showed that Iranian isolates are at the same branch of circulating viruses in the same year compatible with vaccine strains and there was no evidence of a significant distinct subclade of viruses circulating in Iran over the period of study.

Influenza vaccines are the most appropriate tools for preventing influenza infections, therefore the use of appropriate vaccine which has got the highest antigenic similarity to the viruses circulating in the country is very important so the continuous monitoring of the genetic and antigenic characteristics of influenza strains in circulation is an essential policy, not only for optimization of the annual influenza vaccine composition, but also for increasing our knowledge of the molecular epidemiology and evolutionary relations of influenza viruses. The data demonstrated that amino acid changes were limited to some key codons at or near antibody-binding sites A–E on the HA1 molecule. The changes at the antibody-binding site or receptor-binding site are crucial for antigenic drift, and it is important to monitor new H3 isolates for mutations in these sites.

In conclusion, this study has shown conserved sequences as well as some variations due to amino acid substitutions at the receptor-binding site and antigenic sites of the HA gene and some changes in the NA gene of Iranian A/H3N2 strains which generally showed high similarity between them and the vaccine strains used in the northern hemisphere.

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