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Sequence and phylogenetic analyses of neuraminidase gene of Iranian seasonal influenza H1N1 viruses from 2005–2009 and corresponding vaccine strains

N.Z. SHAFIEI JANDAGHI[1], T. MOKHTARI AZAD[1*], S.A. NADJI[2], J. YAVARIAN[1], M. NASERI[1], V. SALIMI[1], K. SAMIMI RAD[1], R. NATEGH

[1]School of Public Health, Tehran University of Medical Sciences, Porsina Ave, Keshavarz Blv. Tehran, Iran, PO 6446; [2]Virology Research Center, NRITLD, Masih Daneshvari Hospital, Shahid Beheshti University of Medical Sciences, Tehran, Iran

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Summary. – Prophylaxis of Influenza A virus infections is based on the vaccines inducing antibodies to the major viral antigens, hemagglutinin (HA) and neuraminidase (NA). Since these antigens continuously change during virus replication in various hosts, only the currently circulating strains should be used in the vaccines. Besides, monitoring of the naturally occurring changes in HA, NA, and respective genes, especially those associated with resistance to the NA inhibitors is necessary. The NA genes of 30 Iranian isolates of influenza H1N1 virus from the seasons 2005–2009 were sequenced and subjected to the sequence and phylogenetic analyses. The seasonal isolates turned out to be closely related to the corresponding vaccine strains, except for the 2007–2008 isolates, which also displayed a higher nucleotide variation. A resistance to the NA inhibitors was found in the 2008–2009 isolates only. The average nucleotide identities of the isolates with corresponding vaccine strains for the years 2005–2009 were 98.83%, 98.55%, 98.7%, 97.55%, and 98.76%, respectively.

Keywords: influenza H1N1 isolates; neuraminidase; phylogeny; drug resistance; Iran

Introduction

Influenza A viruses cause moderate to severe illness and affect people of all ages. Hospitalization and deaths occur mainly in the high-risk groups (Keshtkar-Jahromi *et al.*, 2008). Annual epidemics cause millions cases of severe illness and hundred thousands deaths worldwide (Molinari *et al.*, 2007). The principal targets of the protective immune response against influenza viruses are two major surface glycoproteins HA and NA. Combination of antibodies against these major viral antigens of the circulating strain offers optimal protection against influenza illness (Powers *et al.*, 1996).

In the viral life cycle, NA has enzymatic activity that cleaves sialic acid from complex carbohydrate molecules (receptors), prevents self-aggregation, and facilitates release of virions from the infected cells. It also enables the virus to penetrate mucosal secretion to reach target cells of the respiratory tract (Colman *et al.*, 1994). Immunity induced by NA can reduce the severity and duration of the illness by reduction of the virus replication, which is a reasonable goal in the event of influenza pandemic (Gillim-Ross *et al.*, 2007; Richard *et al.*, 2008; Aoki *et al.*, 2009).

HA and NA genes change periodically due to the sequential evolution in immune or partially immune populations (Munoz *et al.*, 2005). Two important means for the protection and treatment of influenza infection are the vaccination and antiviral drugs. Due to the continuous changes of HA and NA genes, vaccine strains must be selected annually based on the surveillance of influenza viruses circulating in humans. Antiviral drugs, especially NA inhibitors, are the valuable addition to the options used to control the influenza infection ((Moscona *et al.*, 2005; Sheu *et al.*, 2008).

*Corresponding author. E-mail: mokhtari@sina.tums.ac.ir; fax: +98(0)-2188962343. The work place: WHO Influenza Centre, School of Public Health, Tehran University of Medical Sciences.

Abbreviations: HA = hemagglutinin; NA = neuraminidase; PIR = phylogenetically important region

Recent increase in circulating influenza mutants resistant to the NA inhibitors demands a precise surveillance of NA inhibitor-resistant viruses in different countries (Aeron *et al*., 2009; Cheng *et al*., 2009).

In this study, NA genes of 30 Iranian isolates of the influenza H1N1 virus from seasons 2005–2009 were sequenced and compared to the corresponding vaccine strains. NA sequences were subjected to the sequence and phylogenetic analyses with emphasis on the mutation conferring resistance to the NA inhibitors.

Materials and Methods

Clinical samples. The specimens were sent to the National Influenza Centre at School of Public Health of Tehran University from various regions of Iran. Samples were collected in duplicates. One tube was stored at -70°C and the other one was used for screening of the influenza virus A/H1N1 by real-time PCR .The positive samples were inoculated into Madin-Darby Canine Kidney cells (MDCK) for virus isolation. Altogether 30 H1N1 isolates from five recent influenza seasons (2004–2005, 2005–2006, 2006–2007, 2007–2008, and 2008–2009) were available for sequencing. The numbers, names, abbreviations of isolates and Acc. Nos. of their NA complete gene sequences in GeneBank are presented in Table 1.

RT-PCR. RNA was extracted from 150 µl of each cell culture supernatants with NucleoSpin® RNA virus extraction kit (Machery-Nagel Company). cDNAs were synthesized using the M-MLV reverse transcriptase and random hexamer primers. The full length NA gene amplification was carried out using two following sets of forward and reverse primers (designed at NIMR, London,UK) to amplify two overlapping fragments. Set one – N1F1: 5′-AGC AGG AGT TTA AAA TGA ATC CAA-3′and N1R1: 5′-TCT AAG TCT GTT ACT TTT AGT CCT-3′. Set two – N1F2: 5′-ATA ATG ACC GAT GGC CCG AGT AAT-3′ and N1R2: 5′-GTA GAA ACA AGG AGT TTT TTC AAC-3′. The resulting amplicons were sequenced.

Sequence and phylogenetic analyses. Sequences were analyzed with BioEdit version 7.0.0 DNA analysis software (Hall, 1999). Phylogenetic tree was constructed using treecon package version 1.3 b (Van de Peer and De Wachter, 1997) applying Kimura's two-parameter method and the neighbor-joining method with bootstrap analysis (1000 replicates).

Table 1. Designation, abbreviation of isolates, and Acc. Nos. of NA gene sequences in GeneBank

Flu season	Designation of isolate	Abbreviation	Acc. No.
2004–2005	A/Hamedan/7/2005(H1N1)	Ir/7H/05	GU112211
	A/Tabriz/7/2005(H1N1)	Ir/7T/05	GU112212
	A/Tehran/29/2005(H1N1)	Ir/29/05	GU112213
2005–2006	A/Yazd/144/2006(H1N1)	Ir/144/06	GU112216
	A/Azarbayejan-Sharghi/147/2005(H1N1)	Ir/147/05	GU112217
	A/Azarbayejan_Sharghi/150/2006(H1N1)	Ir/150/06	GU112218
	A/Azarbayejan_Sharghi/153/2006(H1N1)	Ir/153/06	GU112219
	A/Azarbayejan_Sharghi/155/2006(H1N1)	Ir/155/06	GU112220
	A/Khorasan/166/2006(H1N1)	Ir/166/06	GU112221
	A/Tehran/171/2006(H1N1)	Ir/171/06	GU112222
	A/Azarbayejan-Sharghi/179/2006(H1N1)	Ir/179/06	GU112223
	A/Azarbayejan-Sharghi/181/2006(H1N1)	Ir/181/06	GU112224
	A/Khorasan/280/2006(H1N1)	Ir/280/06	GU112227
	A/Tehran/371/2006(H1N1)	Ir/371/06	GU112231
	A/Khozestan/380/2006(H1N1)	Ir/380/06	GU112232
	A/Gholestan/215/2006(H1N1)	Ir/250/06	GU112225
	A/Khozestan/459/2006(H1N1)	Ir/459/06	GU112233
2006–2007	A/Tehran/670/2007(H1N1)	Ir/670/07	GU112239
2007–2008	A/Tehran/105/2007(H1N1)	Ir/105/07	GU112214
	A/Hamedan/117/2007(H1N1)	Ir/117/07	GU112215
	A/Tehran/250/2007(H1N1)	Ir/250/07	GU112226
	A/Mazandaran/510/2008(H1N1)	Ir/510/08	GU112234
	A/Khorasan-Jonoobi/511/2008(H1N1)	Ir/511/08	GU112235
	A/Khorasan/512/2008(H1N1)	Ir/512/08	GU112236
	A/Tehran/523/2008(H1N1)	Ir/523/08	GU112237
	A/Khorasan/527/2008(H1N1)	Ir/527/08	GU112238
	A/Esfahan/708/2008(H1N1)	Ir/708/08	GU112240
2008–2009	A/Tehran/310/2009(H1N1)	Ir/310/09	GU112228
	A/Tehran/345/2009(H1N1)	Ir/345/09	GU112229
	A/Tehran/359/2009(H1N1)	Ir/359/09	GU112230

Results and Discussion

In this study, NA genes of 30 Iranian seasonal influenza A/H1N1 isolates collected from February 2005 to January 2009 were completely sequenced. Molecular and phylogenetic analysis was performed to reveal their molecular changes and phylogenetic relationships to the vaccine strains and to investigate NA inhibitor-resistant mutants. Numbering of the isolates was based on N1 gene.

Phylogenetic analysis

Genetic relationships of the NA gene of Iranian isolates with vaccine and reference strains were constructed by neighbor-joining analysis with 1000 bootstrapped replicates. The result showed a continuous evolution of the influenza isolates. The collected isolates were relevant to their vaccine strains except for the isolates collected in the season 2007-08, which showed a higher nucleotide variation. In accordance with the branching of phylogenetic tree, the collected isolates were divided into two groups A, B, and two subgroups BI, BII. These four sets were concurrent with the influenza seasons (Fig. 1).

The three isolates of 2004–2005, fourteen isolates of 2005–2006, and the only isolate of 2006–2007 seasons were clustered in group A with their corresponding vaccine strain. In this group, isolates were similar to the strain A/New Caledonia/20/99 that was recommended as a vaccine strain for the Northern hemisphere in those years. The average similarity to the corresponding vaccine strain for each of the abovementioned seasons was 98.83%, 98.55%, and 98.7%, respectively. We recognized two subgroups in the group B. The 2008–2009 isolates were in subgroup BI with 98.76% average similarity to the vaccine strain A/Brisbane/59/2007. Interestingly, nine isolates of the season 2007–2008 were clustered in subgroup BII with the strain A/Brisbane/59/2007 instead of their corresponding vaccine strain A/Solomon Island/3/2006. The average similarity to these vaccine strains were 99.32% and 97.55% respectively, that showed these viruses are in progression away from the A/Solomon Island/3/2006 (H1N1)-like viruses. In season 2007–2008, two distinct variants of influenza H1N1 viruses were isolated in Netherlands and United States, which resembled A/Solomon Islands/3/06 and A/Brisbane/59/07 strains (Rimmelzwaan *et al.*, 2008; Nelsonn *et al.*, 2008).

The published data from CDC indicated that almost all reported H1N1 isolates in 2007–2008 season were either Brisbane/59/2007-like or Hong Kong/2652/2006-like. Although the strain Solomon Island/3/2006 had been in circulation since 2006–2007, by the time the vaccine was created, it was replaced by the mentioned viruses (http://www.cdc.gov/flu, 2008).

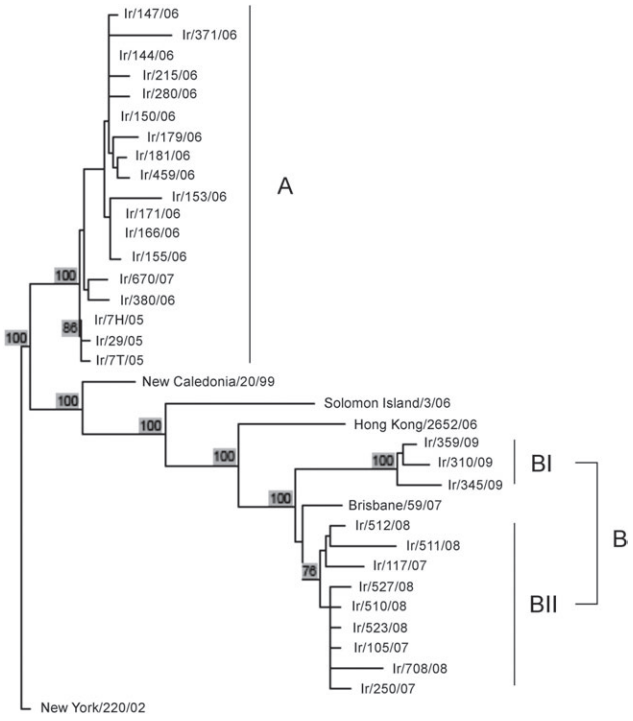

Fig. 1

NA gene-based phylogenetic tree of Iranian influenza H1N1 virus isolates and corresponding vaccine strains

In the abbreviated names of isolates, the last two numbers indicate the year of isolation. Vaccine strains are in boldface.

Sequence analyses

The amino acid analysis showed conserved residues in both catalytic sites (R118, D151, R152, R225, E277, R293, R368, Y402) and framework sites supporting the catalytic residues (E119, R156, W179, S180, D199, I223, E228, H275, E278, N295, E425) for all isolates except H275Y in 2008–2009 isolates (Chutinimitkul *et al.*, 2007; Richard *et al.*, 2008). Viruses in comparison to their corresponding vaccine strains altered amino acids at the antigenic sites (A-C) (Munoz *et al.,* 2005), phylogenetically important regions (PIR) (A-O) and some other residues (Fanning *et al.*, 2000; Bragstad *et al.*, 2008).

In comparison to the strain A/New Caledonia/20/99(H1N1) recommended as a vaccine strain for Northern hemisphere for the seasons 2000–2001 to 2006–2007, all eighteen isolates collected in 2004–2005, 2005–2006, and 2006–2007 showed the mutations E332K at antigenic site C, V48I at PIR-B, and N450D. Besides, the strain Ir/153/06 had the mutation H45N at PIR-B and K78E at PIR-C that are typical of Brisbane/59/2007-like viruses. Furthermore, the mutation S35N was seen in the only isolate collected in the season 2006–2007. For all 2007–2008 isolates, there were found 16 amino acid changes compared to the strain

Table 2. Amino acids substitutions in NA of H1N1 isolates in the season 2007–2008

Isolate name	Amino acid position of NA gene															
	23	45 PIR b	64	77 PIR c	78	157	173	214	222 Ag B	249 PIR f	266	287	329 Ag C	344 Ag C	354 Ag C	452
Solomon Island/3/06	I	H	N	E	K	A	R	E	R	G	T	T	K	D	G	G
Ir/117/07	M	N	H	G	E	T	K	G	Q	K	S	I	E	N	D	D
Ir/250/07	M	N	H	G	E	T	K	G	Q	K	S	I	E	N	D	D
Ir/510/08	M	N	H	G	E	T	K	G	Q	K	S	I	E	N	D	D
Ir/510/08	M	N	H	G	E	T	K	G	Q	K	S	I	E	N	D	D
Ir/511/08	M	N	H	G	K	T	K	G	Q	K	S	I	E	N	D	D
Ir/512/08	M	N	H	G	E	T	K	G	Q	K	S	I	E	N	D	D
Ir/523/08	M	N	H	G	E	T	K	G	Q	K	S	I	E	N	D	D
Ir/527/08	M	N	H	G	E	T	K	G	Q	K	S	I	E	N	D	D
Ir/708/08	M	N	H	G	E	T	K	G	Q	K	S	I	E	N	D	D
New Caledonia/20/99	M	H	H	G	K	A	K	E	R	G	S	T	K	D	G	D
Brisbane/59/07	M	N	H	G	E	A	K	G	Q	K	S	I	E	N	D	D

Ag B, C = antigenic site; PIR B, C, F = phylogenetically important region.

A/Solomon Islands/3/06. Nine of them were typical of A/Brisbane/59/2007-like viruses and the rest of substitutions were found in both groups of A/New Caledonia/20/99-like and A/Brisbane/59/2007-like viruses (Table 2). Compared to their corresponding vaccine strain, all 2008–2009 isolates had the mutations D354G (antigenic site C) and H275Y (oseltamivir resistance mutation), besides N73K in the stalk region of the strains Ir/345/09, and K249G at PIR-F in Ir/310/09 and Ir/345/09. The last substitution was found in both groups A/New Caledonia/20/99-like and A/Solomon Island/3/06-like viruses.

This study showed that the trend of variation in NA gene of H1N1 isolates was going up. Among viruses from five recent flu seasons amino acids substitutions in 2007-08 isolates could have altered antibody binding properties, since epidemiologically important drift variants have four or more amino acids substitutions located in more than one antigenic site (Hata *et al.,* 2006)

Resistance to NA inhibitors

NA inhibitors (zanamivir and oseltamivir) are effective antiviral drugs for the influenza prophylaxis and treatment. The most common genetic indication of oseltamivir drug resistance in N1 is the mutation H275Y. This mutation occurs in or nearby the active site of the enzyme and it was expected that mutant viruses would be less viable than sensitive ones (Dharan *et al.*, 2009).

Until recently, the circulating viruses had shown only low prevalence of NA inhibitors resistance (<1%) (Monto *et al.*, 2006; Dharan *et al.*, 2009). Before the season 2007–2008, the resistant viruses containing the H275Y mutation had been described only in association with the NA inhibitors exposure (Monto *et al.*, 2006). However, recent surveillance revealed a significant number of circulating A/H1N1 influenza strains with the H275Y mutation (Aeron *et al.*, 2009). It is possible that the resistant viruses may have acquired other mutations that compensate these changes in NA gene, so the virus is allowed to continue with the efficient replication and transmission (Helenius *et al.*, 2004; Richard *et al.*, 2008; Cheng *et al.*, 2009).

Analysis of H1N1 viruses in Europe revealed that the overall frequency of H1N1 resistant strains was 25% (Lackenby *et al.*, 2008). In the USA, the resistance to oseltamivir was identified in 98.5% of H1N1 viruses (Dharan *et al.*, 2009). In South Africa, all tested influenza isolates contained the H275Y mutation. Furthermore, these viruses had one or two mutations (M23L and N73K) in the stalk region indicating that some genetic drifts of N1 from the older strains had occurred (Besselaar *et al.*, 2008).

In the present study, all isolates collected from 2005 to 2009 were investigated for the mutation H275Y and for other molecular markers of NA drug resistance: D79G, H126N, S247N, and G249R mutations. The mutation H275Y was found just in all tested isolates of the season 2008–2009 and moreover, one of them had the mutation N73K in stalk region that was found also in African H1N1 viruses (Besselaar *et al.*, 2008).

None of the isolates had a molecular marker of NA drug resistance at the other positions like D79G, H126N, S247N, and G249R.

Glycosylation sites

The specific sequence for N-linked glycosylation is defined as N-X-S/T, where X can be any amino acid except proline or aspartic acid (Helenius *et al.*, 2004). N-linked glycosylation facilitates the folding, stability, solubility, transportation,

antigenicity, and immunogenicity of the protein (Chutinimitkul *et al.*, 2007).

Eight potential glycosylation sites in the NA molecule at the positions 44, 58, 63, 70, 88,146, 235, and 434 (Bragstad *et al.*, 2008) and one additional site in the position 455 have been conserved since 2005 with some exceptions. The additional glycosylation site was created by the S35N mutation in strain Ir/670/07 and in contrast, the H45P substitution eliminated a glycosylation site in strain Ir/147/05.

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