Molecular Analysis of Human Influenza Virus in Tehran, Iran

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Key Words

A/H1N1 · A/H3N2 · Hemagglutinin gene · Influenza virus, Tehran · Phylogenetic analysis

Abstract

Background: To date there are no reports of molecular and phylogenetic analyses of human influenza virus in Tehran, Iran. **Objectives:** We isolated and characterized circulating influenza viruses in a sample of patients in Tehran. **Methods:** Nasal and pharyngeal swabs were collected from 57 individuals who were suspected of having influenza between October 2005 and January 2007. These samples were cultured and subsequently genotyped by RT-PCR and sequencing analyses. **Results:** Twelve of 57 samples (21%) were positive for human influenza virus. Out of the 12 positive samples, 7 were A/H3N2 (58%), 3 were A/H1N1 (25%) and 2 were B subtypes (17%). The phylogenetic analysis of the hemagglutinin gene showed that the H1N1 isolates were close to the A/New Caledonia/20/99 and the H3N2 isolates were close to the A/Panama/2007/99 and A/Moscow/10/99 vaccine strains. **Conclusion:** In a sample of clinical patients in Tehran, Iran, the predominant subtype of human influenza virus was determined to be A/H3N2, followed by A/H1N1 and B. In addition, phylogenetic analysis on H1 showed some genetic drifts from vaccine strains, but the phylogeny of H3 demonstrated that these isolates were from the previous vaccine strains.

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Introduction

Human influenza viruses continually circulate and cause yearly epidemics. Novel strains emerge sporadically as pandemic viruses [1, 2]. The surface hemagglutinin (HA) glycoprotein of influenza viruses is the major target for neutralizing antibodies, and point mutations in the potential antigenic domains of this protein are thought to allow viruses to evade established immune antibodies in the human population. Analyses of epidemic influenza virus isolates, therefore, have chiefly focused on antigenic characterization of the HA glycoprotein in order to detect new variants of each epidemic strain for the recommendation of vaccine strains in each season [3, 4]. Annual epidemics are caused by the rapid evolution of the viral genome. Such changes can be monitored through antigenetic analysis and gene sequencing, particularly the gene that codes for viral HA.

There are a few reports of human influenza viruses from Iran, a seroepidemiological description published in 2004 [5] and a more recent molecular and phylogenetic analysis in Shiraz [6]. Here, we collected a sample of human influenza viruses in Tehran during the 2006–2007 influenza seasons. We used molecular techniques to determine the typing and subtyping of human influenza virus on these samples. The data obtained were then compared to the vaccine strains that were recommended by the WHO for the same period.

Methods

We collected 57 nasopharyngeal swabs from individuals suspected of having influenza. The samples were collected in a clinic and at the influenza unit of the Pasteur Institute in Tehran, Iran, between October 2005 and January 2007.

The MDCK continuous cell line was used to isolate influenza viruses in cell culture. Cells were seeded at a concentration of 5×10^6 cells/ml. After 1 day, 200 μ l of each sample was inoculated into wells containing MDCK cells. The culture medium was then examined for hemagglutinin activity using a 0.5% suspension of chicken erythrocytes.

Viral RNA was extracted from 300 μ l of each sample using a commercial RNX-PlusTM solution (CinnaGen, Tehran, Iran) and immediately frozen at -70° . The cDNA synthesis was performed using SuperScriptTM III first strand synthesis kit (Invitrogen, Carlsbad, Calif., USA) according to the manufacturer's instructions. Amplification of each RNA segment was carried out by RT-PCR, as described previously [6]. The isolates were selected for molecular characterization using bi-directional sequencing. The H1 (543 bp) and H3 (292 bp) fragments from influenza A virus and NS (334 bp) segment from influenza B were used for sequencing and phylogenetic analysis. The PCR products were sequenced in both directions by a CEQTM 2000 dye terminator cycle sequencing kit (Beckman Coulter, Fullerton, Calif., USA) using an automated CEQ 2000XL microcapillary DNA analysis sequencing apparatus (Beckman Coulter). Alignment was performed using Clustal X software, version 1.81 [7].

GenBank accession numbers for sequences determined in this work are EU311401–EU311412.

Phylogenetic trees were constructed using the neighbor-joining method [8]. Bootstrap analysis ($n = 1,000$) was performed to determine the best fitting tree for each gene [9]. Genetic distance was estimated by the Kimura 2-parameter matrix [10]. Molecular Evolution Genetic Analysis (MEGA) computer software, version 2.1 [11], was utilized in this study for phylogenetic and molecular evolutionary analysis and nucleotide differences within and between the isolate sequences.

Results

A total of 12 of 57 samples were found to be positive for human influenza virus (21%). Molecular typing by RT-PCR and subtyping of the positive samples showed 7 as influenza A/H3N2 (58%), 3 as influenza A/H1N1 (25%) and 2 as influenza B (17%).

The nucleotide and amino acid sequences of the HA1 (for type A) and NS (for type B) of the Iran-Tehran isolates were compared with other sequences in GenBank, and with the vaccine strains, during the 2005–2007 influenza seasons.

Among Tehran H1N1 isolates, maximum similarity (99%) was observed with the A/Philippine/67/2004 strain and 95–98% with the A/New Caledonia/20/99 strain. These isolates contained 1–3 amino acid differences compared with A/New Caledonia/20/99 (table 1). In Tehran H3N2 isolates, maximum similarity (99%) was observed with the A/Panama/2007/1999. The 2005-H3N2 isolates showed 81–93% homology to A/California/7/2004 (H3N2)

Table 1. Amino acid substitution of hemagglutinin from H1N1 influenza virus isolates in Tehran compared to vaccine strains

Virus strain	Amino acid position		
	H1N1: 229	252	254
A/New Caledonia/20/99	D	N	N
Tehran/7/2007	G	–	–
Tehran/11/2007	G	Y	S

Table 2. Amino acid substitution of hemagglutinin from H3N2 influenza virus isolates in Tehran compared to vaccine strains

Virus strain	Amino acid position																		
	H3N2: 162	163	172	186	188	189	190	192	193	197	220	222	225	226	227	246			
A/California/7/2004	P	A	E	G	N	N	D	I	S	Q	R	R	D	I	P	N			
Tehran/25/2005	H	L	–	S	D	S	–	–	–	–	S	W	G	V	S	Y			
Tehran/27/2005	H	F	G	S	D	K	E	T	N	R	–	W	G	L	S	Y			
H3N2: 165	172	182	186	189	190	192	193	194	196	197	202	220	222	223	225	226	227	246	
A/Wisconsin/67/2005	N	E	V	V	N	D	I	F	L	A	Q	I	R	R	I	N	I	P	N
Tehran/1/2007	–	–	–	G	–	–	–	S	–	–	–	–	–	–	V	D	–	–	Y
Tehran/6/2007	–	–	–	S	S	–	–	S	I	–	–	V	S	W	V	G	V	S	–
Tehran/9/2007	–	–	–	S	S	–	–	S	I	–	–	V	S	W	V	G	V	S	–
Tehran/14/2007	I	–	–	S	S	–	–	S	I	–	–	V	S	W	V	G	V	S	–
Tehran/16/2007	–	G	I	S	K	E	T	N	–	I	R	V	–	W	V	G	L	S	–

Fig. 1. A neighbor-joining phylogenetic tree of the HA1 gene nucleotide sequence of Iranian isolates and reference gene for H1N1 (a) and H3N2 (b) strains. Bootstrap values based on 1,000 replicates are shown at each main branch. Bootstrap values below 70% are not shown. The scale bar indicates 1% nucleotide divergence. Iranian isolates are marked. ● = Iranian isolates in last study [Ref. 6]; ■ = Iranian isolates in this study; □ = the strains near to Iranian isolates; ▲ = vaccine strains.

and 2006-H3N2 isolates showed 83% to 99% similarity to A/Wisconsin/67/2005 vaccine strains. Alignment of the amino acids of the HA protein demonstrated 10–13 amino acid changes in 2005-H3N2 isolates and 5–15 amino acid changes in 2006-H3N2 isolates, in comparison with candidate vaccine strains (table 2). Tehran B isolates were very close to Florida/02/2006, with 100% homology. As the HA gene was used in vaccine strains, we could not compare Tehran B isolates with vaccine strains.

The 12 positive isolates were selected for phylogenetic analysis. In the H1N1 phylogenetic tree, Tehran isolates were clustered only in the recently discovered isolates of 2000–2006 (fig. 1a). However, in H3N2 phylogenetic tree, some Tehran isolates were clustered with the 1980–1999 strains while others were clustered with the 2000–2006 lineages (fig. 1b). Phylogenetic analysis of Tehran H1N1 isolates confirmed that H1N1 isolates were branched in a unique cluster close to A/New Caledonia-like vaccine virus, with a 77% bootstrap value. The phylogeny of the H3N2 HA nucleotide sequence indicated that Tehran isolates were from the Wisconsin/67/2005 and A/California/7/2004 strains. They were branched in a cluster close to A/Panama/2007/1999- and A/Moscow/10/99-like vaccine strains.

Discussion

The importance of predicting the emergence of new circulating influenza strains for subsequent annual vaccine development cannot be underestimated [12]. On the basis of WHO reports, during the 2005–2006 influenza seasons A/New Caledonia/20/99- (H1N1) and A/California/7/04-like (H3N2) were dominant [13]. Tehran H3N2 and H1N1 nucleotide sequence isolates were compared to the HA1 of other H3N2 and H1N1 reference virus isolates from GenBank. This analysis revealed that Tehran H1N1 isolates were related to the A/New Caledonia/20/99 vaccine strain and clustered in a unique branch. The H3N2

phylogenetic analysis showed that some Tehran H3N2 isolates were closely linked with the Iranian isolates from previous years and some were related to A/Moscow/10/99 and A/Panama/2007/1999 strains.

Amino acid comparison further supported the result of the sequence phylogenetic analysis. Between 1 and 3 amino acid differences in the HA1 protein were observed between Tehran H1N1 isolates and A/New Caledonia/20/99. In 2005-H3N2 isolates 10–13 amino acid changes and in 2006-H3N2 isolates 5–15 amino acid differences were observed when compared with the candidate vaccine strains.

The HA1 subunit of the influenza A protein consists of the globular head and contains 5 major antibody binding sites (A–E). Further analysis will be necessary to better understand the evolution of the mutational changes observed [14]. Wilson and Cox [15] proposed that an epidemiologically important drift variant usually contains 4 or more amino acid substitutions located in 2 or more antigenic sites on HA1 protein. Amino acid substitutions in Tehran H1N1 isolates were not located in these major antigenic sites (table 1). However, the amino acid substitutions in the H3N2 isolates were located in the antigenic sites B and D. It was also confirmed that Tehran H3N2 isolates were generated from the previous vaccine strains.

In conclusion, human influenza A/H3N2, A/H1N1 and B were dominant in a sample of patients in Tehran during the 2006–2007 influenza seasons. In addition, phylogenetic analysis on H1 showed some genetic drift among these samples from vaccine strains, but the phylogeny of H3 demonstrated that these isolates were from the previous vaccine strains generation.

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