IJMS
Vol 36, No 3, September 2011

Original Article

# Phylogenetic Comparison of Influenza Virus Isolates from Three Medical Centers in Tehran with the Vaccine Strains during 2008-2009

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Received: 4 January 2011
Revised: 15 March 2011
Accepted: 24 April 2011

## Abstract

**Background:** Influenza virus is a major infectious pathogen of the respiratory system causing a high degree of morbidity and mortality annually. The worldwide vaccines are decided and produced annually by World Health Organization and licensed companies based on the samples collected from all over the world. The aim of this study was to determine phylogenecity and heterogenecity of the circulating influenza isolates during 2008-2009 outbreaks in Tehran, compare them with the vaccine strains that were recommended by WHO for the same period.

**Methods:** Nasopharyngeal swabs (n=142) were collected from patients with influenza and influenza-like illness. Typing and subtyping of the isolates were performed using multiplex RT-PCR and phylogenetic analysis was carried out for hemagglutinin genes of the isolates.

**Results:** Fifty out of 142 samples were positive for influenza A virus, and no influenza B virus was detected. Phylogenetic analyses revealed that the A/H1N1 isolates were related closely to A/Brisbane/59/2007, and the A/H3N2 isolates were close to A/Brisbane/10/2007 vaccine strains.

**Conclusion:** The findings of the present study demonstrate that the A/H1N1 was the predominant subtype of human influenza virus among the patients studied in Tehran during 2008-2009 winter seasons. In addition, some amino acid variation was found in Tehran/2008/H1N1 isolates from the 2008-2009 vaccine strain, but the H3N2 isolates showed higher genetic resemblance to the vaccine strain.
**Iran J Med Sci 2011; 36(3): 172-177.**

**Keywords** ● Influenza A Virus ● hemagglutinin ● influenza vaccine ● sequence analysis

## Introduction

Influenza viruses are negative-stranded, segmented RNA viruses belonging to the family of Orthomyxoviridae. There are three types of A, B, and C of the virus according to antigenic differences in two of their internal proteins, nucleoprotein (NP) and matrix protein (M).[1] Every year, influenza A and B viral infections cause high levels of morbidity and mortality worldwide.[2] Influenza A viruses are further subdivided into subtypes based on the surface antigens, hemagglutinin (HA) and neuraminidase (NA).[3] To date, 16 HA and 10 NA subtypes have

been found in avian species, but only three HA (H1-H3) and two NA (N1and N2) subtypes have been identified in human.[4] New human subtypes of influenza viruses may emerge through major antigenic changes (antigenic shift) by genetic reassortment of the genome segments of different influenza viruses from diverse animal species in a doubly infected host cell.[5,6] Minor antigenic changes (antigenic drift) are caused by point mutation in viral genome, particularly in surface glycoproteins HA and NA, that are potential antigens of influenza viruses.[7] These changes lead to the emergence of new variants of virus, and result in the annual influenza epidemics.[8]

Since, two subtypes of influenza A (H1N1 and H3N2) and an influenza B viruses are circulating in the community annually, current vaccines are thus trivalent.[9] Each year, World Health Organization (WHO) based on the circulating strains recommends which strains should be used in vaccines for the Northern and Southern Hemispheres.[10] Most protection occurs when the vaccine strains are antigenically similar to the circulating strains.[10,11] Therefore, phylogenetic analysis of circulating influenza strains is necessary to predict the virus antigenic variations, which leads to subsequent epidemic or pandemic.

The aim of this study was the phylogenetic and heterogenetic analysis of prevalent strains of influenza virus in Tehran during 2008-2009 influenza season and compare them with the vaccine strains that were recommended by WHO for the same period.

## Materials and Methods

*Clinical Samples*

The study was approved by the University Ethics Committee, and written informed consent was obtained from all participants. Nasopharyngeal swab specimens were collected from 142 patients suffering from respiratory illness between October 2008 and March 2009. The samples were collected from the Outpatient Clinic of Shahid Beheshti University, diagnostic Influenza Lab of Pasteur Institute of Iran, and Pediatric Infectious Disease Research Center, Tehran. The swabs were placed in viral transport medium (VTM) and centrifuged at 3000 rpm for 20 minutes. The supernatants were separated and stored at -70°C until tested. The VTM contained Minimum Essential Medium (MEM), gelatin, penicillin/streptomycin and amphotericin B.

*RNA Extraction and cDNA Synthesis*

RNA was extracted from 300 µl of each sample using a commercial easy-RED ™ solution (iNtRON, Korea) and eluted in 20 µl DEPC treated water. Complementary DNAs were synthesized using RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Canada) and Random Hexamer primer (5'-3'). Brifly, 10 µl RNA, 1 µl DEPC-treated water, and 1 µl Random Hexamer primer (10 pmol/µl) were mixed and incubated at 70°C for 5 min and immediately cooled on ice. Then, the mixture of 4 µl reaction buffers 5x, 2 µl of dNTP mix (10 mM), 1 µl of Ribolock™ RNase Inhibitor (20 U/ml) and 1 µl of RevertAid™ M-MuLV Reverse Transcriptase (200 U/µl) was added to the tube contained RNA and primer. The tube incubated for 5 min at 25°C followed by 60 min at 42°C and ultimately 5 min at 70°C according to the manufacturer's instructions.

*Polymerase Chain Reaction*

Two different douplex RT-PCR reactions were performed to determine type and subtype of influenza viruses. The RT-PCR methods were optimized previously.[3] Two sets of primers were used to distinguish type of viruses: the matrix protein gene of influenza A virus (M-A) and nucleoprotein gene of influenza B virus (NP-B). Primers designed for the hemagglutinin glycoprotein gene of influenza A/H1N1 and A/H3N2 viruses (H1-A and H3-A) were used for subtyping. These primers were designed from conserved and consensus regions of about 30 different relevant isolates retrieved from GenBank database using multiple alignments.[3] The reaction mixture contained 6 µl of the sample's cDNA, 12.5 µl of master mix containing 1x PCR buffer, 1.5 U Taq polymerase enzyme (CinnaGen), 1.5 mM $MgCl_2$, 0.2 mM dNTPs mix (Fermentas, Vilnius, Lithuania), and 0.5 µM of each appropriate primers (CinnaGen) shown in table 1. Sterile, distilled water was added to reach a final volume of 25 µl. The PCR conditions were 95°C for 5 min, followed by 35 cycles of 94°C for 40 sec, 63°C (for MA and NP-B primers annealing) or 58°C (for H1and H3 primers annealing) for 40 sec, 72°C for 40 sec and a final extension at 72°C for 5 min. Gel electrophoresis of the PCR products using 2% agarose gel and ethidium bromide staining was performed.

*Sequencing and Phylogenetic Analysis*

All 17 subtyped positive samples were assessed for molecular characterization of HA1 gene. Gene sequencing and phylogenetic analysis were carried out for H1 (543 bp) and H3 (292 bp) fragments from influenza A virus. The resulting amplicons of HA1 fragment from

**Table 1:** Primers used for the typing and subtyping of influenza viruses

| Name | Sequence 5'- 3' | Tm | Position | Size (bp) |
|---|---|---|---|---|
| M-A-For | GGTTTTGGCCAGCACTACAGC | 66.4 | 558–578 | |
| M-A-Rev | ACCCCCATTCGTTTCTGATAGG | 65.8 | 737–758 | 201 |
| NP-B-For | ATAGGAAGAGCAATGGCAGACAGAG | 66.5 | 772–796 | |
| NP-B-Rev | ACAAAACTCTCAGGTCTTCATAGGCAG | 66.4 | 1175–1204 | 429 |
| H1-A-For | CCGACTATGAGGAACTGAGGGAG | 57.8 | 335–375 | |
| H1-A-Rev | TCGCATCACATTCATCCATTG | 56.3 | 857–877 | 543 |
| H3-A-For | TGAACGTGACTATGCCAAACAATG | 58.5 | 547–570 | |
| H3-A-Rev | CGTATTTTGAAGTAACCCCGAGGA | 59.2 | 815–838 | 292 |

H1 and H3 genes of the isolates were cleaned up followed by sequencing in both directions which was performed on ABi 3730x1 genome analyser (Source BioScience, UK). Alignment of H1 and H3 gene sequences from Iranian isolates with about 60 H1 and H3 gene sequences as reference was performed by CLUSTALX software, version 1.81.[12] Genetic distance was calculated using the Kimura two-parameter matrix.[13] The neighbor-joining method was used to construct phylogenetic trees.[14] Bootstrap analysis (n=1,000) was performed to confirm the reliability of phylogenetic tree.[15] Molecular Evolution Genetic Analysis (MEGA) computer software, version 4,[16] was utilized in this study for phylogenetic and molecular evolutionary analysis and nucleotide differences within and between the isolate sequences.

*Nucleotid GenBank Accession Numbers*

The nucleotide sequences determined in this study have been submitted to GenBank under the following accession numbers: HM346544 -HM346560.

## Results

The molecular typing and subtyping of the isolates revealed that 50 out of 142 samples were positive for human influenza A virus. Out of those 50 positive samples, 15 were H1N1 and only 2 H3N2 were detected. No influenza type B was identified in this study. Agarose gel electrophoresis of RT-PCR products are shown in figure 1 and 2. The sensitivity cut-off of RT-PCR was 0.1 ng of total template RNA genome as described previously.[17]

*Sequence and Amino Acid Analysis*

All 17 influenza A positive samples were sequenced. The nucleotide and deduced amino acid sequences of the HA1 from 17 isolated samples were compared with other GenBank sequences as well as with current vaccine strains. Based on nucleotide alignments, the Tehran/2008/H1N1 isolates had maximum similarity (98.5%) with New South Wales/18/99 isolates and 98% with those of Auckland/176/99, New Caledonia/20/99 and Tehran/7/2006. In the alignment generated based on the HA1 portion amino acid sequences, Tehran/2008/H1N1 isolates demonstrated 4-6 amino acid differences compared with vaccine candidate strain A/Brisbane/59/2007 (table 2). The Tehran/2008/ H3N2 isolates showed maximum similarity (100%) with the Nagasaki/N03/2005 strain and 99% with the Brisbane/10/2007. Alignment of the amino acids of

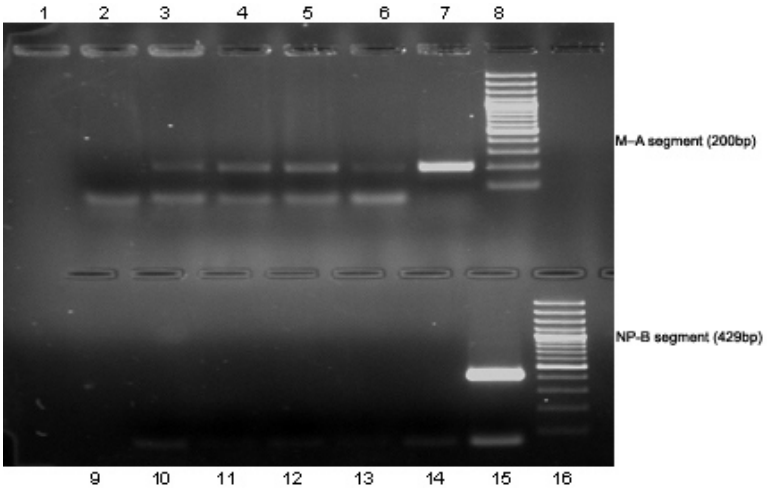


**Figure 1:** Agarose gel electrophoresis of RT-PCR products for influenza typing. Lane1: Negative control, Lane 2-6 & 9-14: clinical samples, Lane 7: influenza type A, Lane 15: influenza type B, Lane 1 & 10: Gene Ruler 100bp (CinnaGen, Iran).

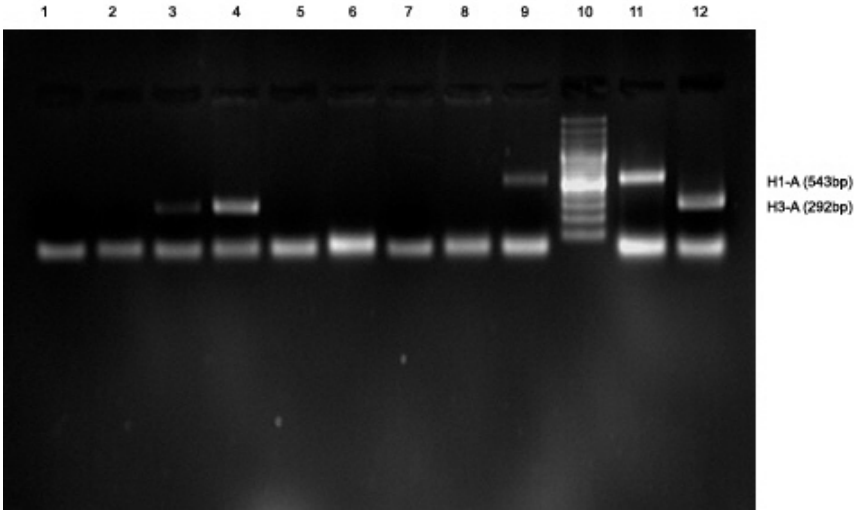


**Figure 2:** Agarose gel electrophoresis of RT-PCR products for influenza A virus subtyping. Lane 1: Negative control, Lane 2-9: clinical samples, Lane 10: Gene Ruler 100bp (CinnaGen, Iran), Lane 11: A/H1N1, Lane 12: A/H3N2.

**Table 2:** Amino acid substitutions of hemagglutinin gene from Tehran/2008/H1N1 isolates compared with the vaccine strain (A/Brisbane/56/2007)

| Virus strain | Amino acid position | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 152 | 167 | 192 | 210 | 213 | 233 | 235 | 248 | 278 | 285 |
| A/Brisbane/56/2007 | N | E | A | N | I | S | K | D | R | R |
| Tehran/30/2008 | - | K | V | - | N | - | R | - | W | - |
| Tehran/31/2008 | - | K | V | T | N | - | R | - | W | - |
| Tehran/32/2008 | - | K | V | T | N | - | R | - | W | - |
| Tehran/35/2008 | K | K | V | - | D | - | R | - | W | - |
| Tehran/36/2008 | - | K | V | T | N | - | R | - | W | - |
| Tehran/38/2008 | - | K | V | T | N | - | R | - | W | - |
| Tehran/39/2008 | - | K | V | T | N | - | R | - | W | - |
| Tehran/40/2008 | - | K | V | - | N | - | R | - | W | - |
| Tehran/42/2008 | - | K | V | T | N | - | R | - | W | - |
| Tehran/43/2008 | K | K | V | - | D | - | R | - | W | - |
| Tehran/70a/2008 | K | K | V | - | D | - | R | - | W | - |
| Tehran/12a/2008 | - | K | V | - | D | - | R | G | W | K |
| Tehran/31a/2008 | - | K | V | - | D | - | R | - | W | - |
| Tehran/2a/2008 | - | K | V | T | N | I | R | - | W | - |
| Tehran/53a/2008 | - | K | V | - | N | - | R | - | W | - |

the HA protein from these isolates demonstrated one amino acid change with the vaccine strain A/Brisbane/10/2007 (table 3).

**Table 3:** Amino acid substitutions of hemagglutinin gene from Tehran/2008/H3N2 Isolates compared with the vaccine strain (A/Brisbane/10/2007)

| Virus strain | 210th amino acid |
|---|---|
| A/Brisbane/10/2007 | Proline |
| Tehran/13a/2008 | Lysine |
| Tehran/16a/2008 | Lysine |

*Phylogenetic Analysis*

Nucleotide sequence of the HA1 region of the Tehran/2008/H1N1 and Tehran/2008/H3N2 isolates were compared with the vaccine strains and other influenza viruses, and their genetic relationships were considered by neighbor joining analysis with 1000 bootstrapped replicates. These analyses revealed that our H1N1 isolates were linked with A/Brisbane/59/2007 vaccine strain and also with the Iranian isolates from previous years that all clustered in a distinct clade with 98% bootstrap value (figure 3a). Moreover, phylogenetic analysis showed that our H3N2 isolates and Nagasaki/N03/2005 strain branched in a unique cluster close to A/Brisbane-like vaccine virus, with a 99% bootstrap value (figure 3b). The phylogenetic tree is available at:

http://ijms.sums.ac.ir/images/userfiles/Sep%2011/fig1a.jpg
http://ijms.sums.ac.ir/images\userfiles\Sep 2011\fig1b.jpg

**Figure 3:** Phylogram of the HA1 region of the HA gene nucleotide sequence of Iranian H1N1 (a) and H3N2 (b) isolates and reference genes. The tree was created by neighbor joining method and bootstrapped with 1000 replicates. ▲,■ and ● denote Iranian isolates in previous studies, Tehran/2008 isolates in this study and vaccine strains, respectively.

## Discussion

For subsequent annual vaccine development, the analysis of circulating influenza virus strains and detection of antigenic changes is necessary worldwide. Annually, WHO recommends the most suitable composition of influenza vaccine strains for the forthcoming influenza season based on surveillance data gathered in the world.[18] On the basis of WHO reports, the influenza vaccine used in the Northern hemisphere during 2008-2009 contained H1N1 (A/Brisbane/59/2007) and H3N2 (A/Brisbane/10/2007) strains.[19]

There are a few serological and molecular reports of human influenza viruses from Iran. Serological studies on the distribution of human influenza viruses in Iran from 1999 to 2001 have demonstrated that the annual patterns of Iranian isolates were identical to those reported worldwide.[20] With regard to the present study, the A/H1N1 was predominant subtype of human influenza virus among Iranian patients in Tehran during 2008-2009 winter season. This result was confirmed with the Center for Disease Control (CDC) report, which showed the predominance of H1N1 subtype.[21]

Five major antigenic sites (A-E) are identified on the surface of the HA1 subunit of hemagglutinin protein of influenza A virus. An epidemiologically important drift variant usually contains four or more amino acid substitutions located in two or more antigenic sites on HA1 protein.[22] Molecular and phylogenetic analysis of human influenza virus isolates in Shiraz during 2003-2004 seasonal outbreaks showed a few genetic drifts from samples of vaccine strains that were recommended by WHO for the same period. Moreover, the amino acid sequence analysis exhibited that substitutions of amino acid in the H1N1 and H3N2 isolates were not located in antigenic sites on HA1 protein.[3] The nucleotide and amino acid sequence analyses of Iranian isolates in Shiraz during 2005-2007 influenza outbreaks revealed that most of H3N2 isolates varied at least in two out of five major antigenic sites from A/California/7/2004 vaccine strain. In contrast, H1N1 isolates showed a notable antigenic and sequence resemblance to A/New Caledonia/20/99 vaccine strain.[23]

The circulating strains of human influenza virus in Tehran were further studied during 2005-2007 influenza seasons. Influenza A/H3N2, influenza A/H1N1 and influenza B was determined as predominant subtypes, respectively. Amino acid comparison of the H1N1 isolates with the New Caledonia vaccine strain showed 1-3 amino acid substitutions in positions other than HA1 antigenic sites. In 2005-H3N2 isolates 10-13 amino acid differences and in 2006-H3N2 isolates 5-15 amino acid changes were observed in comparison with A/California/7/2004 and A/Wisconsin/67/2005 vaccine strains. These amino acid substitutions were located in the antigenic sites B and D. Based on phylogenetic analysis, H1N1subtypes showed some genetic drifts from vaccine strain but H3N2 subtypes were from the previous vaccine strains.[24]

The present study showed that out of 50 positive isolates for human influenza A virus, 15 and 2 strains were H1N1 and H3N2, respectively. Nucleotide sequences of these 17 isolates were compared to the HA1 gene of other H1N1 and H3N2 reference virus isolates in GenBank. The H1N1 isolates were genetically close to A/Brisbane/59/2007 vaccine strain and Iranian isolates from previous years. Ten H1N1 isolates were clustered in a distinct branch close to New Caledonia/20/99 strain, and five of them were branched with two Tehran/2006 isolates (figure 3a). These subtypes were different from A/Brisbane/59/2007 vaccine virus in 5-7 amino acids whose substitutions were located in the antigenic sites B and D. The phylogenetic analysis of H3N2 HA nucleotide sequences demonstrated our H3N2 isolates were related to the A/Brisbane/10/2007 vaccine strain and cluster in a unique branch (figure 3b). These isolates varied from vaccine strain only in one amino acid, which was located in the antigenic site D. Further analysis will be necessary to estimate the evolution of the mutational changes in the antigenic sites on the HA1 protein.[25]

## Conclusion

Human influenza A/H1N1 was predominant subtype during 2008-2009 influenza seasons in Tehran. In addition, some amino acid variations were found in Tehran/2008/H1N1 isolates from the 2008-2009 vaccine strain, however, the H3N2 isolates showed higher genetic resemblance to the vaccine strain.

## Acknowledgement

We would like to thank Dr. Mazaheri and Ms. Shokati of Pasteur Institute of Iran, and Dr. Tafazzoli and Dr. Mofid of the Outpatient Clinic of Shahid Beheshti University for their assistance in the collection of samples.

**Conflict of Interest:** None declared

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