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Original Article

Molecular Characterization and Phylogenetic Analysis of Neuraminidase Gene in A/H1N1 Influenza Virus Isolates Circulating in Iran, 2014-2015

Molavi Z[1], Behzadian F[1*], Fotouhi Chahooki F[2], Moeini S[1]

1. Research center for Biosciences and Biotechnology, Malek-Ashtar University,Tehran- Iran.
2. Influenza Research Lab, Pasteur Institute of Iran, Tehran, Iran.

Abstract

Background and Aims: Influenza is one of the most important emerging and reemerging infectious diseases in the world. The aim of this study is molecular and phylogenetic analyses of the variations in circulating influenza A/H1N1 virus isolates during 2014-2015 in Iran and investigate on the drug resistance conditions in the related Iranian isolates.

Materials and Methods: Throat samples from Iranian patients with acute respiratory tract infection were subjected for typing and subtyping by multiplex real-time RT–PCR. Seven positive samples were randomly selected and full-length amplification of Neuraminidase gene (NA) were carried out by RT-PCR. The related amplicons were sequenced and analyzed by bioinformatics software.

Results: Phylogenetic analysis on the NA gene of the A/H1N1 isolates revealed a high degree of sequence identity with the corresponding NA genes from viruses circulating in the USA, Russian, India, Thailand, and East Asia region. Moreover, the NA sequences showed point mutations N44S, V106I, V241I, N248D, N369K; resulted in increasing of stability and transmission improvement of the viruses [1, 2]. The NA sequences showed a similarity of 98-99% with the reference strain NC_026434.1 /California/2009. 52.5% of mutations were silent. On amino acid level, the dedicated sequence of NA protein showed 97% identity among isolates. Oseltamivir and Zanamivir resistant mutations were not detected in the isolates.

Conclusions: Gathering NA sequence data of influenza viruses isolated from Iran and compare it with counterpart data from other geographical regions would be helpful to explain epidemiological rules governing antigenic drift and reveal the antiviral drug (neuraminidase inhibitors) sensitivity of human influenza A (H1N1) viruses.

Keywords: Influenza A/H1N1 virus, Phylogenetic analysis, Neuraminidase, Iranian isolates.

Introduction

Influenza virus is an epidemic infectious agent which belongs to the Orthomyxoviridae family [3]. Of all common respiratory illnesses, influenza has the most dramatic effect on communities. Up to now, three types of influenza viruses including A, B, and C are known upon immunological and biological properties [4, 5]. Influenza A viruses are zoonotic pathogens and subdivided into two antigenic determinants of Hemagglutinin (HA) and Neuraminidase (NA)[6, 7]. To date, eighteen different HA antigens (HA1 to HA18) and eleven NA antigens (NA1 to NA11) have been serologically identified [8] . Among the above mentioned viruses, three major human influenza viruses (A/H1N1, A/H3N2, and B) are circulating globally [9].

[*] **Corresponding author:** Research center for Biosciences and Biotechnology, Malek-Ashtar University,Tehran- Iran, Email: fbehzadian@yahoo.com.

HA and NA are both surficial glycoproteins which are embedded in the envelope and are targeted by human humoral immunity [7, 10]. Whereas the HA is the main receptor-binding site that mediates viral attachment to the cell membrane and contributes to membrane fusion, the NA removes the cell surface receptor (sialic acid) and has a critical role in the release of progeny virus particles as well as the spread of virus from host to host [11] .

Vaccines play a critical role in immunity and provoke activation of humoral immunity against influenza viruses [12]. Nevertheless, the impression of this intervention could be eliminated receiving a mismatch mutation compare to seasonal influenza vaccine which leads to inability of immune response. Therefore, antiviral agents could be an alternative in prevention and control of influenza virus [13, 14] . Currently, in addition to the adamantanes there are three neuraminidase inhibitor (NI) drugs licensed worldwide; Tamiflu (oseltamivir), Relenza (Zanamivir) and Rapivab (Peramivir). Many substitutions observed as NI drugs resistant [15] .

Molecular analysis of influenza A viruses, particularly the HA and NA molecular feature of circulating strains are important for determining the virus epidemic impact and for the selection of appropriate vaccine strain. Neuraminidase inhibitors were found to cause potent and selective inhibition of all human influenza viruses [16, 17] . NA genome sequence data also reveals mutation profile associated with neuraminidase inhibitor (Laninamivir, Oseltamivir, Peramivir, and Zanamivir) resistance [18-20] .

Here we characterized the NA gene segments of influenza A (H1N1) viruses which randomly isolated from Iranian patients during 2014 seasonal outbreak. The analysis performed on the basis of phylogenetic topology, genetic clustering and pairwise amino acid variations.

Methods

Isolation of Influenza viruses. Influenza viruses were isolated by standard methods from nasal swabs of patients with clinical symptoms at the Pasteur Institute of Iran, during fall and winter 2014. Isolated viruses had been tested by a real-time RT-PCR assay to detect and the Influenza A to H1N1 and H3N2 subtypes. Seven high titer H1N1 samples were randomly selected and propagate on MDCK cells.

Amplification of NA gene. Viral RNA was extracted from 200 µl of the supernatant of cell cultures for each sample using "High pure viral RNA kit" (Roche, Germany) and eluted in 20 µl DEPC treated water. Specific primers for amplification of target sequence (1413nt of NA segment), were designed from conserved region of neuraminidase gene using the BioEdite software (version 7.2.5) ; 5'-AGTAGAAACAAGGAGTTTTTT-3' as reverse and 5'-CCAAACCAAAAGATAATAACC-3' as forward primers.

Reverse Transcription was done using AMV Reverse Transcriptase (Cat. No. 12328-019, Invitrogen) according to the manufacturer's instruction. The primer Uni12; 5'-AGCRAAAGCAGG-3', [21] which is complementary to the conserved 3'end of all influenza A virus RNA segments was used for cDNA synthesis. High Fidelity Taq DNA polymerase (Cat. No. 11304-029, Invitrogen) was used for amplification according to the manufacturer's instruction.

The PCR first optimized through a gradient experiment then the samples were amplified under the following profile: Initial denaturation at 94 ºC for 2 min and 35 cycles (94 ºC for 20 sec, 56.6 ºC for 30 sec, and 68 °C for 1.5 min) concluded by a final extension step at 68 °C for 10 min. PCR products were analyzed by gel electrophoresis on a 1.5% agarose gel and purified by a GF-1 PCR Clean-up Kit (Vivantis, Malaysia) according to the manufacturer's instruction prior to sequencing.

Sequencing. The primers used for sequencing were the same used for amplification of NA fragment. Sequencing was done in both directions using a BigDye® Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) and ABI Prism® 3700 DNA analyzer sequencer (Applied Biosystems) at Sequence Laboratories of

FirstBase company, Malaysia. All sequences have been deposited in the GenBank database under accession numbers; from KY859964 to KY859970

Phylogenetic analysis. Phylogenetic trees of NA genes were constructed using the Maximum-likelihood (ML) method within MEGA 6.0 using Kimura's two-parameter model. The stability of nodes and tree branching was determined by bootstrapping using 1000 repeats of drawing.

Results

As demonstrated in Figure 1 the gel electrophoresis result showed the best annealing temperature of 56.6 for NA amplification. Figure 2 also show the result of PCR carried on the samples from different patients. The formation of 1410 bp amplicons compared to the DNA molecular weight marker has been shown.

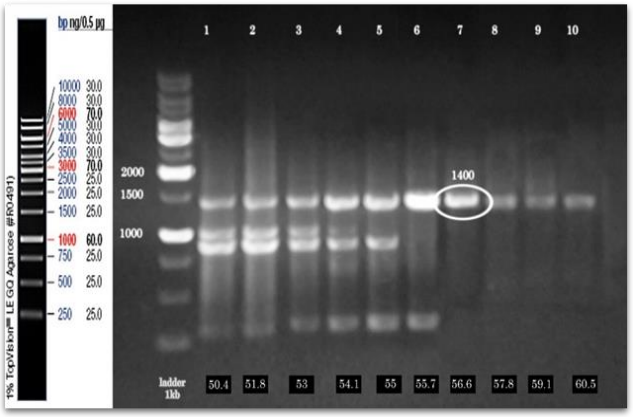

Fig. 1. A gradient PCR was performed at annealing temperatures spanning 50°C to 60°C. Estimates of the temperatures achieved are as follows (the numbers for the unnumbered lanes apply to the lanes from left to right, respectively): 50.4°C, 51.8°C, 53°C, 54.1°C, 55°C, 55.7°C, 56.6°C, 57.8°C, 59.1°C, 60.5°C. A 1000-kb ladder is found in lanes 1.

Neuraminidase inhibitor resistant (NAI-resistant) substitutions were checked in 7 sequences obtained in this study. No influenza A (H1N1) viruses has shown noteworthy mutations caused NAI resistant as shown in table 1 Therefore, all strains were sustained.

In parallel, the coding region of Neuraminidase sequences were aligned with reference,

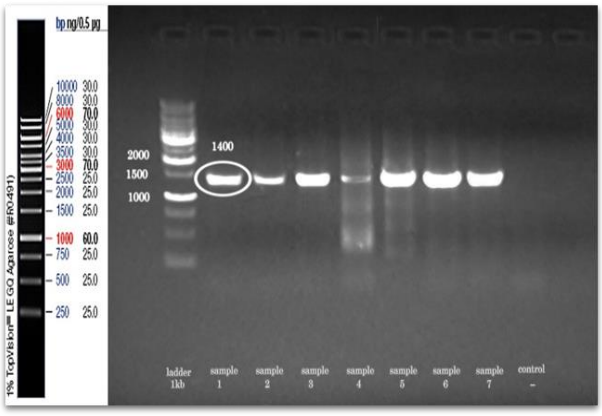

Fig. 2. Agarose gel electrophoresis of the neuraminidase (NA) gene. Result of PCR amplification of NA gene 7 virus samples isolated from Iranian patient. A fragment of approximately 1410 bp was amplified using primers designed. A 1000-kb ladder is found in lanes 1and negative control (water instead of template) in last lanes.

Eurasian and American sequences. The tree produced base on nucleotide and protein sequences using the maximum likelihood (ML) by MEGA6 software. The unrooted phylogenetic tree showing the familiarity between 7 NA sequences, including references, based on full length Neuraminidase sequence is shown in fig 3 One cluster was formed by sixth Iranian strains obtained in this research and strains from USA, Thailand, Japan, South Korea, India and Russia. One other case study strain formed the second cluster with strains from Japan, China, Sweden, Canada, Malaysia and USA.

Phylogenetic analysis using the sequences obtained in this study and reference strains from GenBank clustered all viruses into 2 main well-supported clades and 4 sub-clades which most of samples were present in clade A in sub-clade A-a, which contains most of sequences, some mutations such as; N44S, V241I, V264I, N270K, N369K and N386K have observed. In sub-clade A-2a T16I recognized. In clade B, reference strain and ARJ63935 strain are in two different sub-clades. In most of clade B strains, V106I was noticed. 18 (number) mutations were detected in amino acid sequences of 7 strains induce antigenic variations, as shown in table 2.

Table 1 : Phenotypic NAI susceptibilities of 7 clinical A(H1N1) virus isolates and vaccine strain virus

	NA sequences protein ID	Mutations occurred at active sites													Phenotype(s) in NA Sequence
		116	117	119	136	149	156	199	223	247	275	278	295	223 , 297	
Vaccine strain	**ACQ63272.1**	V	I	E	Q	V	R	D	I	S	H	E	N	IH	Sensitive
Sample 1	**ARJ63935**	V	I	E	Q	I	R	D	I	S	H	E	N	IH	Sensitive
Sample 2	**ARJ63936**	V	I	E	Q	I	R	D	I	S	H	E	N	IH	Sensitive
Sample 3	**ARJ63937**	V	M	E	Q	I	R	D	I	S	H	E	N	IH	Sensitive
Sample 4	**ARJ63938**	V	M	E	Q	I	R	D	I	S	H	E	N	IH	Sensitive
Sample 5	**ARJ63939**	V	M	E	Q	I	R	D	I	S	H	E	N	IH	Sensitive
Sample 6	**ARJ63940**	V	M	E	Q	I	R	D	I	S	H	E	N	IH	Sensitive
Sample 7	**ARJ63941**	V	M	E	Q	I	R	D	I	S	H	E	N	IH	Sensitive

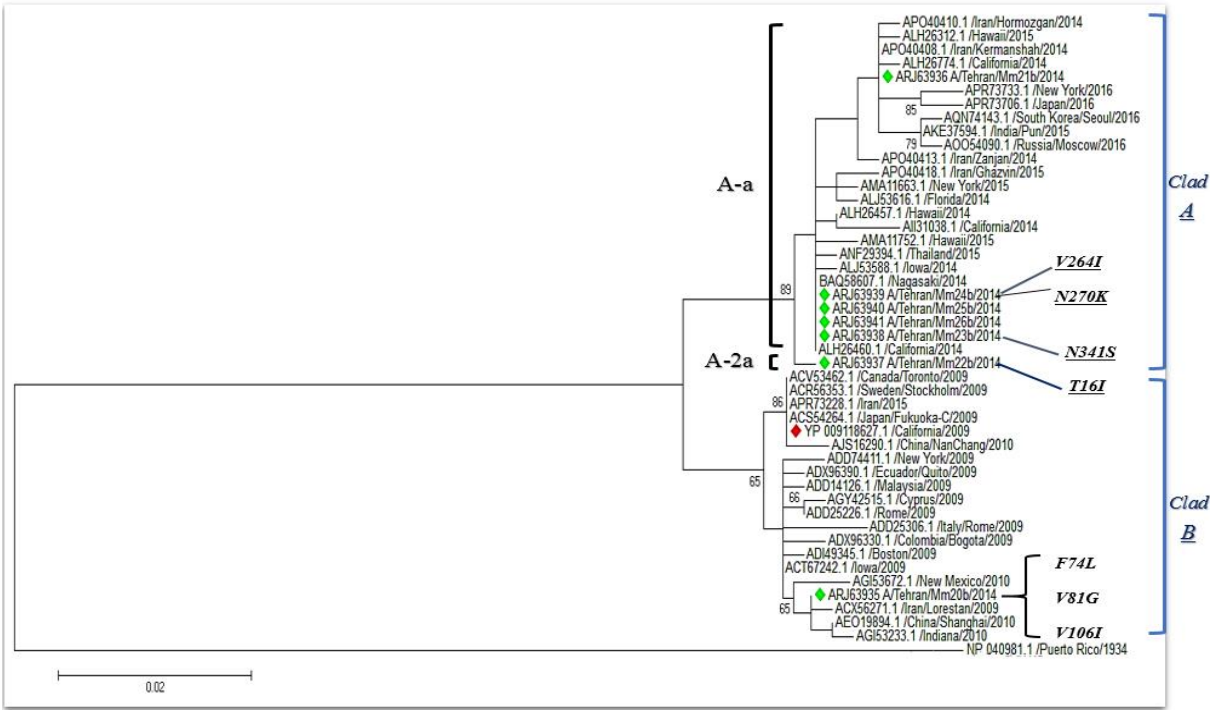

Fig. 3. Phylogenetic tree of the neuraminidase (NA) gene segment 6 of influenza A(H1N1) viruses at the protein level. Phylogenetic tree constructed on the basis of the NA H1N1 influenza viruses collected in Iran from 2014 to 2015. The trees were constructed using the maximum likelihood (ML) method with bootstrap analysis of 1,000 replicates . The NAs sequences of Iran H1N1 isolates were compared with relevant virus sequences available in GenBank. sequences from representative influenza A(H1N1) isolates from Iran and closest blast hits from different regions of the world. Sequence from vaccine strains [NC_026434.1 /California/2009)] were also included. Iranian's samples virus isolated are displayed by green signs.

Table 2: Comparison of gene sequences of 7 Iranian H1N1 viruses isolated with vaccine strain H1N1[NC_026434.1 /California/2009)] at amino acid positions in NA gene.

position	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7
16			T/I				
34		I/V	I/V	I/V	I/V	I/V	I/V
40		L/I	L/I	L/I	L/I	L/I	L/I
44		N/S	N/S	N/S	N/S	N/S	N/S
67		V/I					
74	F/L						
81	V/G						
106	V/I						
117			I/M	I/M	I/M	I/M	I/M
200		N/S		N/S	N/S	N/S	N/S
241		V/I	V/I	V/I	V/I	V/I	V/I
248	N/D	N/D	N/D	N/D	N/D	N/D	N/D
264		V/I					
270		N/K					
321		I/V	I/V	I/V	I/V	I/V	I/V
341				N/S			
369		N/K	N/K	N/K	N/K	N/K	N/K
386		N/K	N/K	N/K	N/K	N/K	N/K
432		K/E	K/E	K/E	K/E	K/E	K/E

Discussion

Comprehension of evolution and molecular properties is invaluable in regional health service surveying. Human influenza virus is circulating worldwide annually and causes seasonal epidemics [22]. Phylogenetic analysis is necessary to trace the virus. In this study, phylogenetic analysis of human influenza neuraminidase (NA) genes which received from Iranian patients, indicated the most evolution similarity to USA, East Asia (i.e. India, Japan, Korea, China and Thailand), Russia, Ecuador and Italy. Phylogenetic tree divided into two main clade, clade A and B. Most of Iranian case study strains and 2014-2016 strains are in clade A whereas a case study strain with ARJ63935 A/Tehran/Mm20b/2014 accession number accompanies with vaccine strain and 2009-2010 strains in clade B. Therefore, the origin of most case study strains is worldwide epidemics.

Finally, eighteen single point mutations in seven case study strains leading to amino acid substitutions in antigenic sites compared to the vaccine strain (accession no. NC_026434.1 /California/2009) have been observed. Of the 7 Iranian case study strains, 5 strains which carry N44S, V241I, N369K and N386K mutations placed in A-a subclade. Substitution of asparagine to serine at amino acid position 44 of the above mentioned mutations lead to gain new glycosylation sites which could be increase antigenic properties [23].

Six of seven strains contain mutations leading replacing asparagine with lysine at residue 369, which contributes to increase virus fitness in conjunction with H275Y and increased gene expression and neuraminidase activity in resistant strains [24]. Replacing asparagine with lysine in the 386 amino acid positions in 6 strains have been detected. This substitution cause increased gene expression and neuraminidase activity [25].

Recent studies showed the N369K and V241I substitutions in oseltamivir resistant viruses led to increased proliferation and transmission [1, 26]. These amino acid replacements distinguished in 6 case study strains without H275Y substitution.

Subclade A-a involved six Iranian cases study strains. V264I and N270K substitutions in experimentally determined epitopes of

neuraminidase detected while the effect of mentioned substitutions have not reported to date. Most of strains in clade B, carried V106I substitution. The effects of this substitution is not yet determined in influenza virus biology.

Altogether, N42S, N44S, N369K, N386K and dual V241I+N369K amino acid substitutions occurred in 85.71%. It is noteworthy, that the 52.5% of mutations at the nucleotide level were silent mutations which did not change the amino acid.

According to our surveys done, mutations associated with NA inhibitor drugs resistance was not observed.

In summary, in this study we performed phylogeny relationships, sequencing and molecular analyses of 7 H1N1 influenza viruses isolated from Iran 2014. According to the results shown and discussed, evolutionary pressure engendered towards development of compatibility and stability and increase the enzymatic activity and virus transmissibility. Furthermore, mutations in experimentally determined epitopes may lead to promote the evasion of host immune responses or generation of vaccine resistant strains.

Due to the high mutation rate of neuraminidase, continued monitoring is important to detect changes which lead to increase the virulence, pathogenesis and the wide spread of the H1N1 viruses.

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