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ORIGINAL ARTICLE

Characterization of the neuraminidase genes from human influenza A viruses circulating in Iran from 2010 to 2015

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Abstract
Background Characterization of influenza viruses is critical for detection of new emerging variants. Herein, we analyzed the genetic diversity and drug susceptibility of the neuraminidase gene (NAs) expressed by influenza A/H1N1pdm09 and A/H3N2 viruses circulating in Iran from 2010 to 2015.
Methods We genetically analyzed the NAs of 38 influenza A/H1N1pdm09 and 35 A/H3N2 isolates.
Results The Iranian A/H1N1pdm09 viruses belonged to seven genogroups/subgenogroups, with the dominant groups being genogroups 6B and 6C. The A/H3N2 isolates fell into six gneogroups/subgenogroups, with the dominant genogroups being 3C and 3C.2a. The most common mutations detected among the A/H1N1pdm09 viruses included N44S, V106I, N200S, and N248D. All H1N1pdm09 viruses were genetically susceptible to the NAIs. However, one A/H1N1pdm09 virus from the 2013–2014 season possessed an NA-S247N mutation, which reduces the susceptibility to oseltamivir. In case of H3N2, none of the analyzed Iranian strains carried a substitution that might affect its susceptibility to NAIs.
Conclusion The ongoing evolution of influenza viruses and the detect of influenza viruses with reduced susceptibility to NAIs warrants continuous monitoring of the circulating strains.

Keywords Influenza · A/H1N1pdm09 · A/H3N2 · Molecular genetic analysis · Antiviral resistance · Iran

Introduction

Taxonomically influenza A viruses (IAV) belong to the *Orthomyxoviridae* family of RNA viruses. IAV possesses a single-stranded, eight-segmented RNA genome of negative polarity, which typically encodes 11 or 12 viral proteins [1]. IAVs can be further subtyped according to the antigenic properties of two viral surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA). So far, 16 HA and 9 NA subtypes have been identified [2]. Of these, influenza A/H1N1 and A/H3N2 subtypes are associated with seasonal influenza outbreaks [3]. Amino acid substitutions at important positions on the HA and NA proteins can modify antigenic characteristics and antiviral drug sensitivity of influenza viruses [4]. Along with influenza B viruses, IAVs impose a significant health burden due to the high morbidity and mortality rates associated with their infections [5].

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High genetic diversity also exists within the H1N1 and H3N2 subtypes. Different strains can arise as a result of point mutations and/or reassortment of genome segments. Consequently, new mutant strains constantly emerge and replace the old ones [6]. Immunological pressure and natural selection drives genetic diversity within the HA proteins, whereas drug selection as well as natural selection shape genetic changes in the NA protein. As a result, new resistant populations occasionally evolve and may become predominant as they develop optimal viral fitness [7, 8].

Neuraminidase inhibitors (NAIs) are a class of influenza antivirals that are currently the drugs of choice for treatment of influenza A/H1N1pdm09 and A/H3N2 infections [9–11]. Mutations in the NA enzyme catalytic site or framework are usually associated with resistance to NAIs [11–13]. A histidine to tyrosine mutation at NA residue 275 (H275Y, N1 numbering) is most commonly associated with resistance to oseltamivir and peramivir but not zanamivir among both pandemic H1N1 2009 (H1N1pdm09) and H3N2 viruses [10]. Other mutations, such as E119V, I223K/R/V, S247N, and N295S (N1 numbering) in H1N1pdm09 influenza virus and E119V, Q136K, D151E, I222V, R224K, E276D, N249S, R292K, and R371K (N2 numbering) in H3N2 influenza virus have been also described to induce resistance to oseltamivir and/or zanamivir [14–17].

In this study, we investigated the genetic diversity and drug susceptibility of the *NA* genes from influenza A/H1N1pdm09 and A/H3N2 viruses circulating in Iran from 2010 to 2015.

Materials and methods

Virus isolates

The methodology for sample collection and screening has been described previously [18]. During the 2014-2015 influenza season, respiratory samples were obtained from Iranian patients diagnosed with influenza-like symptoms from 3 provinces of Iran (Markazi, Semnan, and Zanjan) and were transported to the National Collaborating Laboratory of Influenza, Pasteur Institute of Iran. Collected samples were tested by real-time reverse transcription polymerase chain reaction (RT-PCR) using the WHO protocol [19, 20]. Virus from positive samples with Ct values less than or equal to 20 were propagated and isolated in MDCK cells. Hemagglutination assays (HA) were performed using a 0.5% suspension of chicken erythrocytes to confirm virus growth. Positive cultures were harvested and stored at -70 °C until further analysis could be performed. A total of 11 isolates (5 A/H1N1pdm09 and 6 A/H3N2) with a HA titer ≥4 (passage no. 1 or 2) were selected from the 2014-2015 season [18].

Complete nucleotide sequences of the *NA* genes of Iranian influenza A/H1N1pdm09 and A/H3N2 viruses that were reported between 2010 and 2015 were obtained from the Influenza Resource Database (IRD; http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html) and the Epiflu platform using the Global Initiative on Sharing All Influenza Data (GISAID; http://platform.gisaid.org/) databases.

RNA extraction, RT-PCR, and DNA sequencing

A Roche High Pure Viral RNA Extraction Kit (Roche, Germany) was used for extraction of RNA from the positive culture supernatants. The NA genes of 5 A/H1N1pdm09 and 6 A/H3N2 virus isolates previously identified during surveillance in Iran [18] were amplified using a QIAGEN One-step RT-PCR Kit (Qiagen, Hilden, *Germany*) with the primers listed in Table 1. Amplification products were analyzed in a 1.5% agarose gel and were further purified using the GF-1 PCR Clean-up kit (Vivantis, Malaysia). DNA sequencing was performed by an ABI sequence Genetic Analyzer (Applied Biosystems, Foster City, CA) at First BASE Laboratories Sdn Bhd (Selangor, Malaysia). The NA sequences of these isolates were submitted to GenBank under the accession numbers shown in Table 2.

Sequence alignment and phylogenetic analysis

The NA sequences generated in this study were edited and assembled using Chromas Lite v.2.5.1 (Technelysium Pty Ltd., Australia) and CLC sequence viewer v.6.7. For sequence analysis, the NA sequences of WHO influenza A/H1N1pdm09 and A/H3N2 vaccine strains (2010-2015), available on the Influenza Research Database (http://www.fludb.org/), were used as reference strains.

Table 1 Sequences and positions of the primers used to amplify the coding regions of the NA genes from the A/H1N1pdm09 and A/H3N2 subtypes, by one-step RT-PCR

Influenza Subtype	Primer sequence (5'-3')	Target	Position (nt)	PCR product (bp)
H1N1	F: GGTCTGTATGACAATTGGAAT R: CACCGTCTGGCCAAGACC	NA	56-76 1384-1401	1350
H3N2	F: GCAGGAGTAAAGATGAATCC R: CCTGACAATGTGCTAGTATG	NA1	8-27 809-828	821
H3N2	F: CATGCGATCCTGACAAGTGTTATC R: TTCTAAAATTGCGAAAGCTTATAT	NA2	375-398 1412-1435	1061

Table 2 The A/H1N1pdm09 and A/H3N2 influenza isolates analyzed in this study

Subtype	Strain	Collection date (DD/MM/YYYY)	Accession numbers Segment 6 (NA gene)
A/H1N1pdm09	A/Iran/91755/2014	25/11/2014	KY643830
	A/Iran/91874/2014	26/11/2014	KY643829
	A/Iran/814/2014	01/12/2014	KY643828
	A/Iran/802/2014	02/12/2014	KY643827
	A/Iran/791/2014	03/12/2014	KY643826
A/H3N2	A/Iran/91247/2014	22/11/2014	KY643835
	A/Iran/91244/2014	25/11/2014	KY643836
	A/Iran/92243/2014	01/12/2014	KY643833
	A/Iran/92246/2014	03/12/2014	KY643832
	A/Iran/116/2014	04/12/2014	KY643831
	A/Iran/92172/2014	05/12/2014	KY643834

The phylogenetic analysis included globally representative sets of A/H1N1pdm09 and A/H3N2 sequences from human influenza viruses that were reported to the NCBI Influenza Resource Database (http://www.ncbi.nlm.nih.gov/genomes/FLU) and the Global Initiative on Sharing All Influenza Data (GISAID; http://platform.gisaid.org/) databases. The ClustalX v.2.1 program was used for alignment of all nucleotide and protein sequences. The maximum likelihood value and Akaike information criterion indicated that the T92+G model was the best fit model for the *NA* gene analysis. A phylogenetic tree for the NA-coding nucleotide sequences was generated by Molecular Evolutionary Genetic Analysis (MEGA) v.6.06 [21] using a maximum likelihood tree and the T92+G model with 1,000 bootstrap replicates. Clades were defined using WHO-designated reference strains [22]. The deduced amino acid sequences were analyzed for key residues that associate with resistance or reduced susceptibility to NAIs that were summarized by the WHO GISRS antiviral susceptibility expert working group (AVWG) [23].

Results

The aim of our study was to assess the genetic diversity and to determine the frequency of NAI resistance among *NA* genes from Iranian A/H1N1pdm09 and A/H3N2 viruses. The nucleotide sequences of the *NA* genes from 38 influenza A/H1N1pdm09 and 35 A/H3N viruses collected in Iran during 2010 through 2015 were analyzed, including those sequenced for this study.

Molecular evolution of the *NA* genes of influenza A/H1N1pdm09 viruses

The nucleotide sequences of the *NA* genes from 38 influenza A/H1N1pdm09 virus isolates were analyzed, including eleven isolates from the 2010-2011 season, nine isolates each from the 2012-2013, the 2013-2014, and the 2014-2015 seasons.

The NA phylogenetic tree representing the 38 Iranian A/H1N1pdm09 isolates, along with globally representative A/H1N1pdm09 strains, is shown in figure 1. Seven various genogroups have evolved during the past few years. The A/California/07/2009 vaccine strain is presented in genogroup 1 [24, 25]. Phylogenetic analysis of the A/H1N1pdm09 viruses circulating in Iran between 2010 and 2015 revealed seven genogroups/subgenogroups with the dominant strains being genogroups 6B (13 isolates) and 6C (9 isolates) (Fig. 1).

During the 2010-2011 influenza season, A/H1N1pdm09 viruses of different genogroups (3, 5, and 7) circulated in Iran (Fig. 1, Supplementary Table 1). Three of the 11 Iranian isolates clustered in genogroup 3 (represented by A/Hong Kong/3934/2011) along with viruses from England, Singapore, and the United States and were characterized by V106I/N248D/V394I substitutions. Four isolates contained additional V241I and N369K substitutions and clustered in genogroup 5 (represented by A/Astrakhan/1/2011). Also four variants were detected with an N44S substitution and clustered with Denmark and Thailand isolates in genogroup 7 which is represented by the reference strain A/St. Petersburg/100/2011.

No Iranian isolates were submitted to the NCBI and GISAID databases during the 2011-2012 influenza season. During the 2012-2013 season, the majority of viruses clustered in genogroups 6A and 6C. Out of the nine investigated isolates from this season, four A/H1N1pdm09 viruses were genetically related to the A/Massachusetts/10/2013 reference strain (representative strain of genogroup 6C). These isolates clustered with viruses from the EMR including Jordan and Oman and were characterized by I106V/N200S changes. The I106V substitution is a reversion to the A/California/07/2009-like vaccine strain. The I106V

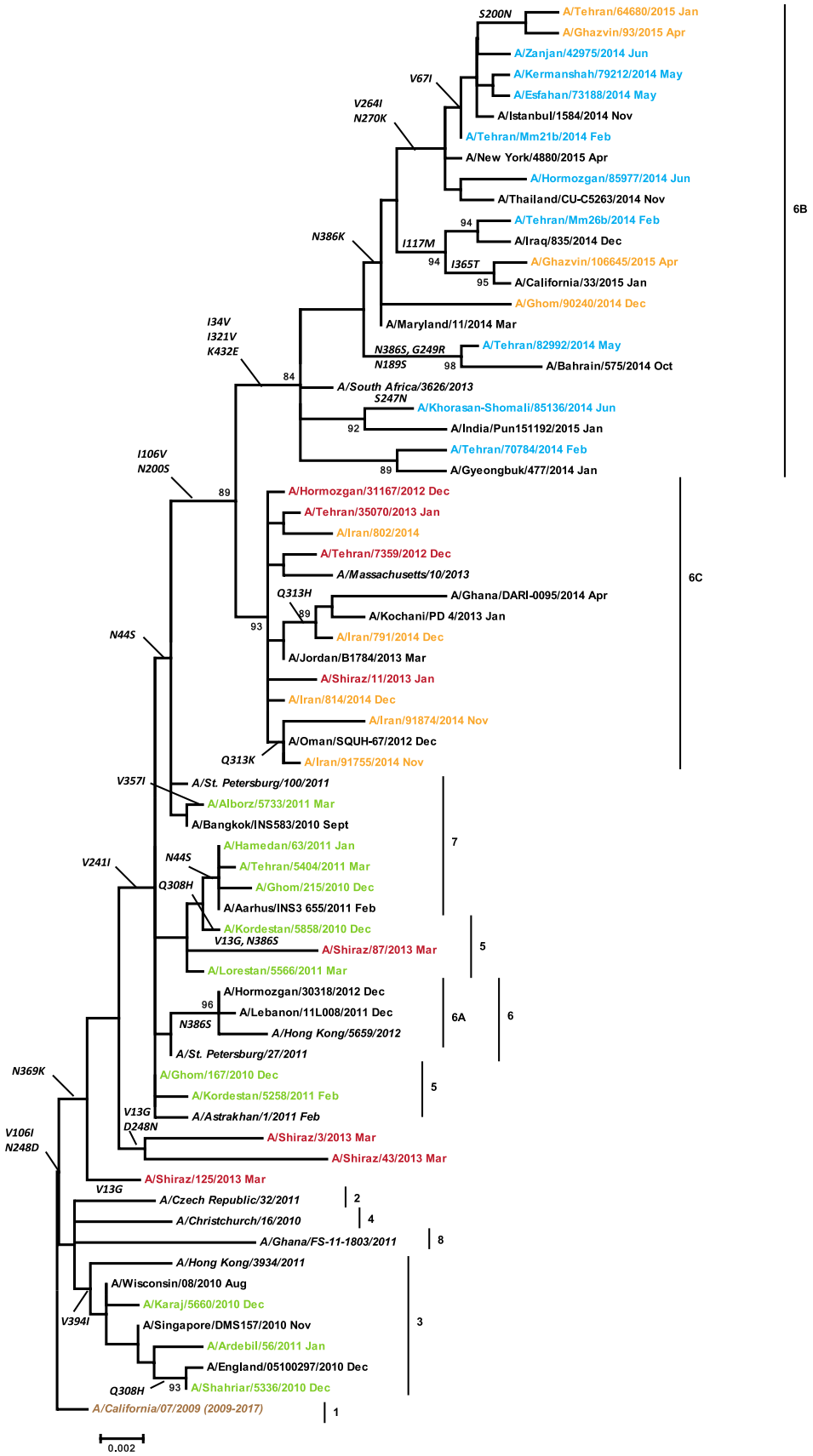

Fig. 1 Phylogenetic trees constructed for the *NA* gene (1350 nucleotides) of H1N1pdm09 influenza viruses collected in Iran from 2010 to 2015. The trees were constructed using the maximum likelihood (ML) method with bootstrap analysis of 1,000 replicates. The A/California/07/2009 vaccine strain was used as the root for the tree and bootstrap values greater than 75% are shown. The sample collection seasons are colored as follows: 2010–2011 (green), 2012-2013 (red), 2013-2014 (blue), 2014–2015 (orange). The vaccine strain is in bold brown and reference strains are in bold black. The representative strain of each clade is shown in italics. Amino acid mutations characteristic of the main clusters or the Iranian isolates are indicated on the branches (color figure online)

and N200S substitutions were fixed within the subsequent emerging genogroup 6B and 6C viruses. One Iranian isolate from the 2012-2013 season had an N386S substitution and was related to the reference strain A/Hong Kong/5659/2012 (genogroup 6A). This cluster also included a Lebanese isolate from the earlier season. Another Iranian isolate from the 2012-2013 season grouped in genogroup 5 with viruses isolated in the 2010-2011 influenza season. Three other Iranian isolates from the 2012-2013 season selected a V13G substitution and were distinct from the defined genogroups. An N248D substitution has been conserved among Iranian isolates since 2009; however, this mutation was absent in two isolates from the 2012-2013 season.

During the 2013–2014 season, only one genogroup (6B) of viruses was detected in Iran that was characterized by the I34V/I321V/K432E substitutions, similar to the reference strain A/South Africa/3626/2013. The 2013-2014 Iranian isolates clustered together with other strains isolated from South Korea and the United States from the same season. Two unique mutations, V264I and N270K, were identified in the NA sequences of the 2013-2014 A/H1N1pdm09 viruses analyzed in this study, consistent with the strains circulating in India and the countries of south-eastern Asia [26]. Isolates belonging to genogroup 6B also maintained the I106V and N200S substitutions. Viruses isolated in Iran during the 2014-2015 season belonged to two phylogenetic groups. Four out of the nine Iranian isolates clustered in genogroup 6B along with viruses collected from the same season in the EMR, including isolates from Bahrain and Iraq. The five other isolates belonged to genogroup 6C and clustered in the same genogroup with other Iranian A/H1N1pdm09 viruses isolated in the 2012-2013 influenza season. Among these five viruses, 3 contained Q313H/K substitutions in their NA proteins.

The V241I and N369K amino acid substitutions were reported to improve stability of the NA protein and to enhance virus fitness [27]. These two mutations were observed in 89.47% (34/38) of the Iranian A/H1N1pdm09 viruses isolated during 2010 through 2015. An N386S or N386K mutation was detected in most of the 2013-2014 and 2014-2015 Iranian isolates. Substitutions H275Y and N295S confer NAI-resistance in influenza A/H1N1pdm09 viruses [25]. None of these classical mutations were detected in any of the 38 Iranian H1N1pdm09 isolates analyzed in this study (Supplementary Table 1). Only one Iranian virus (A/Khorasan-Shomali/85136/2014) isolated in 2013-2014 season was found to carry the S247N mutation. This substitution can interfere with the stability of the neuraminidase inhibitor in the enzyme-active pocket and result in reduced susceptibility to oseltamivir [8]. None of the analyzed Iranian A/H1N1pdm09 strains carried the I223R substitution, which confers reduced susceptibility to both oseltamivir and zanamvir and elevated levels of resistance to the NAIs in combination with the H275Y substitution [23, 28]. The Q136K mutation, associated with reduced susceptibility to zanamivir (Relenza) and peramivir [29], was not detected in any of the Iranian H1N1pdm09 isolates. A combination of NA-Q313R and -I427T mutations was associated with resistance to both oseltamivir and zanamivir [23, 30]. These dual mutations were not detected in any of the Iranian H1N1pdm09 viruses during our study period. None of the H1N1pdm09 isolates possessed the E119V amino acid substitution, associated with resistance to oseltamivir [31].

Molecular evolution of the *NA* gene in influenza A/H3N2 subtypes

The nucleotide sequences of the *NA* genes from 35 influenza A/H3N2 virus isolates were analyzed, including one isolate from the 2010-2011 season, thirteen isolates from the 2011-2012 season, three isolates from the 2012-2013 season, six isolates from the 2013-2014 season, and thirteen isolates from the 2014-2015 season.

Seven genogroups and several subgenogroups have been defined among circulating H3N2 viruses [32]. Phylogenetic analysis indicated that the Iranian isolates fell into six genogroups/subgenogroups, with the dominant groups being genogroup 3C (19 isolates) and subgenogroup 3C.2a (11 isolates) (Fig. 2). Only one NA sequence from the 2010-2011 season in Iran was available from the Epiflu platform in the GISAID database. This isolate possessed the D127N, I307M, L338F, and N402D mutations and belonged to genogroup 1, represented by A/Norway/1186/2011 (Fig. 2, Supplementary Table 2). Viruses isolated during the 2011-2012 season belonged to different groups. Four out of the 13 Iranian isolates from this season were characterized by four mutations (Y40C, I176M, and R210K, N402D) and belonged to genogroup 5 (represented by A/Alabama/05/2010) along with other strains that were isolated from the United States in the same season. Six of the 13 isolates belonged to subgenogroup 3B (represented by A/Athens GR/112/2012) that also harbored viruses from Greece, Jordan, Thailand, and the United States. One isolate was clustered with strains isolated from Jordan and Kyrgyzstan and belonged to the subgenogroup 3C.1 represented by the A/Victoria/361/2011, the WHO-recommended vaccine strain for the 2012-2013 and 2013-2014 seasons. Two other isolates from the 2011-2012 season clustered together and were distinct from the established genogroups. One of these viruses contained an amino acid substitution at position 197 (D197N). The S367N and K369T mutations appeared towards the end of the 2011-2012 season and were later conserved in all of the Iranian isolates from the three subsequent seasons. Only one isolate from the

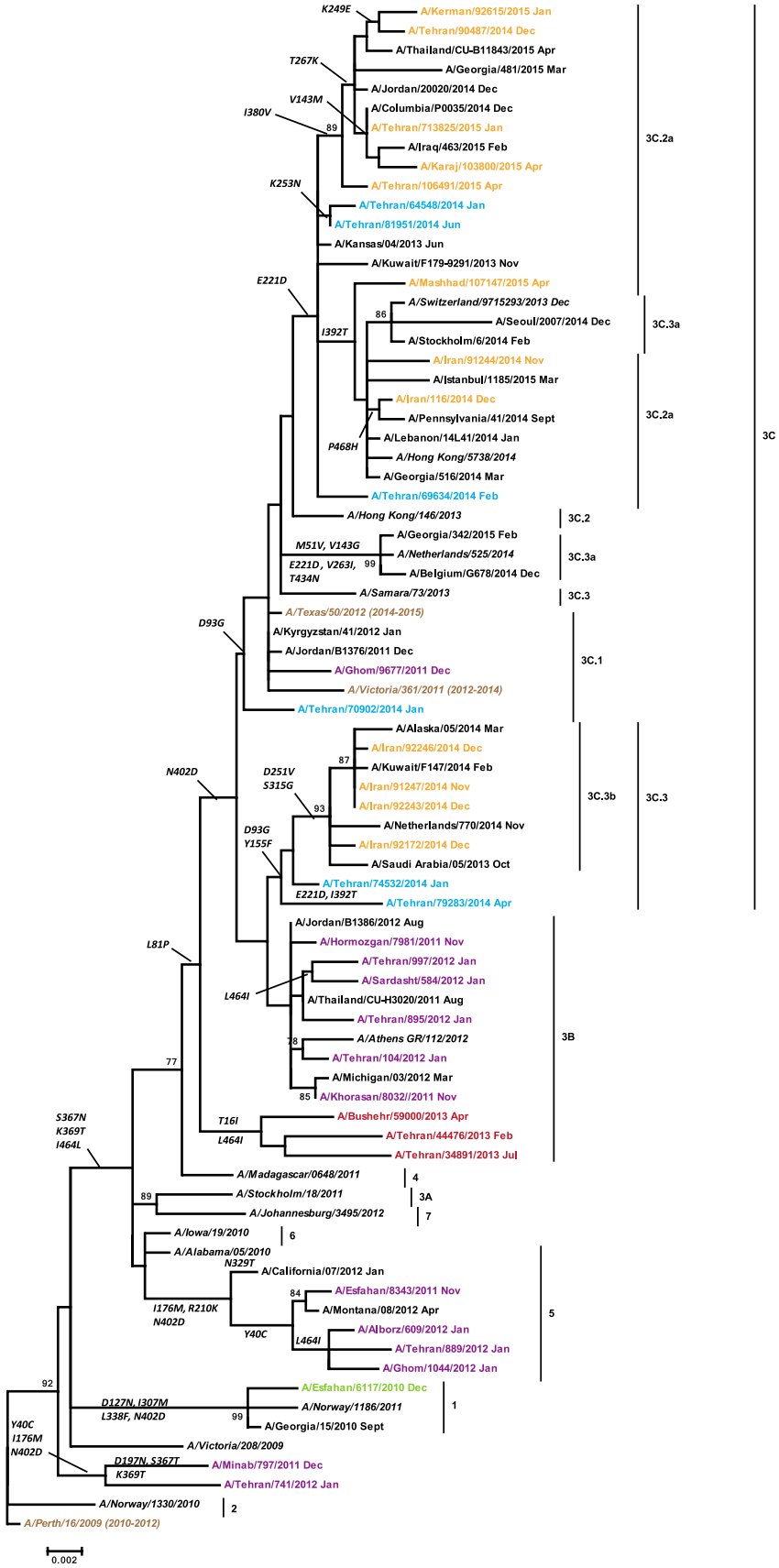

Fig. 2 Phylogenetic trees constructed for the *NA* gene (1410 nucleotides) of H3N2 influenza viruses collected in Iran from 2010 to 2015. The trees were constructed using the maximum likelihood (ML) method with bootstrap analysis of 1,000 replicates. The A/Perth/16/2009 vaccine strain was used as the root for the tree and bootstrap values greater than 75% are shown. The sample collection seasons are colored as follows: 2010–2011 (green), 2011-2012 (purple), 2012-2013 (red), 2013-2014 (blue), 2014–2015 (orange). The vaccine strains are in bold brown and reference strains are in bold black. The representative strain of each clade is shown in italics. Amino acid mutations characteristic of the main clusters or the Iranian isolates are indicated on the branches (color figure online)

2011-2012 season lacked the S367N and K369T substitutions and one isolate, A/Minab/797/2011, had an S367T mutation. During the 2012–2013 season, three Iranian A/H3N2 viruses clustered together in subgenogroup 3B. Amino acid mutation, L18P, has been conserved in Iranian isolates since 2012-2013 season; however, this mutation was present in seven isolates from 2011-2012 season. The 2012-2013 isolates possessed a T16I that was only found in the viruses isolated during this season.

Iranian isolates from the 2013-2014 season formed different groups in the phylogenetic tree. One isolate selected the D93G mutation and belonged to the subgenogroup 3C.1 represented by the 2 A/H3N2 vaccine strains, A/Victoria/361/2011 and A/Texas/50/2012. Three isolates had an additional E221D mutation and clustered in sub-subgenogroup 3C.2a along with isolates from Kuwait and the United States. Two other isolates possessed the Y155F substitution in addition to the D93G mutation and clustered in subgenogroup 3C.3, which harbored isolates from Kuwait, Saudi Arabia, and the United States from the same season.

The 2014-2015 viruses belonged to two subgenogroups. Four isolates selected the four (D93G, Y155F, D251V, and S315G) mutations and clustered in subgenogroup 3C.3b that also harbored viruses from Kuwait, Saudi Arabia, the Netherlands, and the United States from the same and earlier seasons. Viruses carrying these NA substitutions have their corresponding HA genes in genogroups 3C.3 and 3C.3b [18]. *NA* genes of viruses with *HA* genes in the 3C.2a subgroup [18] fell into two distinct clusters. Both clusters shared the E221D substitution. One cluster was defined by the NA I392T substitution and contained three Iranian A/H3N2 viruses along with viruses from the Lebanon, Turkey and the United states from the same and earlier seasons. The other cluster was characterized by the substitution I380V and contained five Iranian viruses along with viruses from the EMR including Iraq and Jordan. Mutations V143M, K249E, T267K, and I380V were selected during the 2014-2015 season, and have not been previously detected in Iranian isolates. The amino acid substitutions, N402D+I464L, were detected among all of the Iranian isolates from the 2013-2014 and 2014-2015 seasons. The combination of these substitutions was previously detected in seven Iranian isolates from the 2011-2012 season. The D93G substitution was found among all of the 2013-2014 and 2014-2015 isolates. Two isolates in 2013-2014 and four isolates in the 2014-2015 seasons carried the double mutation D93G+Y155F in their NA proteins and were classified in genogroup 3C.3. The substitutions H150R and E221D are adjacent to the catalytic sites of the NA protein. All of the 2013-2014 and 2014-2015 A/H3N2 viruses possessed the H150R mutation; however, the substitution E221D was found in four isolates in 2013-2014 and eight isolates in the 2014-2015 influenza seasons.

The *NA* gene sequences of all 35 Iranian H3N2 viruses were analyzed for mutations that have been associated with reduced susceptibility or resistance to NAIs (Supplementary Table 2). None of the investigated A/H3N2 strains had the E119A/I/V/D/G, D151E, R292K, or N294S mutations, which are associated with reduced susceptibility or resistance to oseltamivir [23, 33, 34]. Moreover, none of the Iranian isolates possessed the Q136K substitution, which confers resistance to zanamivir [17]. None of the isolates selected the R224K, E276D, or R371K mutations, which have been associated with resistance to both oseltamivir and zanamivir [35].

Discussion

According to WHO reports, influenza A/H1N1pdm09 and A/H3N2 viruses were alternatively detected as the predominant viruses during 2010 through 2015 in Iran [18, 36–40]. During the 2010-2011, 2012-2013, and 2014-2015 seasons influenza A/H1N1pdm09 was the most commonly detected virus in the Middle East and neighboring countries in Asia with relatively little A/H3N2 or type B [36, 38, 40]. However, in the 2011-2012 and 2013-2014 seasons, influenza A/H3N2 subtype was detected as the predominant virus in these regions of the world [37, 39].

In the present study, detailed molecular analysis of the *NA* genes of 38 influenza A/H1N1pdm09 and 35 A/H3N2 Iranian virus isolates (2010-2015) was carried out.

Characteristics of the Iranian A/H1N1pdm09 viruses

Influenza A/H1N1pdm09 viruses have evolved and undergone critical genetic changes since their emergence [22]. During 2010 to 2015, the A/H1N1pdm09 viruses collected in Iran revealed variation in the circulation of different genetic groups, suggesting multiple introductions of this virus into Iran. Between 2010 and 2015, most of the Iranian viruses fell into genogroups 6B and 6C.

Substitutions N44S, V106I, N200S, V241I, N248D, and N369K [41] were the most common mutations detected among the Iranian A/H1N1pdm09 viruses during 2010 through 2015. The V241I and N369K substitutions were found in 89.47% (34/38) of the Iranian H1N1pdm09 viruses. These substitutions were reported to increase the transmission fitness through compensation of the reduced NA activity in the H275Y oseltamivir resistant strains [42]. Therefore, these strains might accommodate the H275Y mutation without any cost to virus transmissibility. 94.73% (36/38) of the Iranian A/H1N1pdm09 viruses carried an N248D mutation and 36.84% (14/38) possessed the V106I/N248D substitutions in their *NA* genes. The V106I and the N248D mutations are associated with the enhanced low-pH

stability of the NA of H1N1pdm09 viruses and might have contributed to the rapid worldwide spread and adaptation of this virus to humans [43].

The mutation N44S may affect the antigenic properties of influenza viruses by changing the N-glycosylation potential of the related motif at positions 42–44 from N-Q-N to N-Q-S [44]. This mutation locates in the stalk of the NA and was detected in 71.05% (27/38) of the A/H1N1pdm09 Iranian viruses isolated during the study period. A K432E substitution, located in an antigenically important region, was detected in clade 6B Iranian viruses as well as viruses from other countries such as China [45]. The I34V+I321V+K432E mutations were detected among all of the 2013-2014 Iranian isolates clustered in genogroup 6B. Previous studies showed that this combination started to arise in December 2012, however, no functional role has been experimentally identified for these NA mutations [46, 47].

Influenza A/H1N1pdm09 viruses with mildly reduced susceptibility to oseltamivir and zanamivir were detected in the Asia-Pacific region in 2011, due to an NA-S247N mutation [48]. This novel variant was detected in more than 10% of community specimens in Singapore and more than 30% of samples from northern Australia during the early months of 2011 [48]. The S247N mutation is predicted to interfere with the stability of the NAI in the enzyme-active pocket, resulting in reduced susceptibility and in extremely high resistance, when combined with the H275Y mutation [8, 48]. During the 2013-2014 influenza season, only one Iranian A/H1N1pdm09 virus strain possessed the NA-S247N mutation. This mutation was previously detected in an isolate from nearby Lebanon during the 2010-2011 season [8]. The prevalence of the S247N mutation in this study was 0.02%, which was lower than that reported in Thailand (1.4%) and other countries [48, 49].

Characteristics of the Iranian A/H3N2 viruses

Phylogenetic analysis of the Iranian A/H3N2 NA sequences revealed variation in the circulation of different genogroups during 2010-2015. In our study, viruses clustered in genogroup 3 predominantly circulated during the study period. 54.3% (19/35) of the Iranian isolates clustered separately in clades 3C.2 and 3C.3 and were characterized by amino acid mutations at positions L81P, D93G, S367N, K369T, N402D, and I464L. The S367N+K369T mutations appeared towards the end of the 2011-2012 season in Iran. 91.4% of Iranian A/H3N2 isolates carried the dual substitutions S367N and K369T in their NA, which result in the addition of a potential N-glycosylation site at residue 367. Similar substitution patterns have been reported elsewhere in the world [49, 50]. The addition of a potential N-linked glycosylation site might affect the antigenic properties of influenza viruses [50]. The mutations Y40C, I176M, R210K were detected in isolates from the 2011-2012 influenza seasons that lacked the S367N and K369T substitutions. Previous studies also detected these mutations in clade 1 A/H3N2 viruses isolated after 2009 [51]. The L81P+D93G dual substitutions were detected among Iranian isolates during the 2013-2014 and 2014-2015 seasons. These mutations were previously reported in influenza A/H3N2 viruses circulating in India during 2013 season [50]. According to a previous study on A/H3N2 isolates from southern China, the accumulation of HA (D69N, Y110H, I246V, E296A/T, A214S, V239I and N328S) mutations along with NA gene mutations L81P and D93G, resulted in antigenic drift that possibly gave rise to the A/H3N2 influenza epidemic during the 2011-2012 season [52]. In our study, the D93G+Y155F+D251V combination mutations were detected along with an S315G substitution during the 2014-2015 season. *In vitro* NAI testing indicated that these mutations do not confer reduced susceptibility to NAIs [50, 53].

The NA amino acid substitution N402D appeared in 2010-2011 and was carried by 91.4% of the Iranian A/H3N2 isolates. This mutation results in the loss of an N-linked glycosylation site in the viruses clustered in the clades 3B and 3C [49]. The T267K mutation that was observed among the Iranian A/H3N2 viruses was previously reported in Ukraine during the 2014-2015 season [54]. The positively selected sites (267 and 464) in the NA segment of influenza viruses may represent antigenic sites [13, 55].

This study contributes to our understanding of the ongoing evolution of the NA proteins of influenza A/H1N1pdm09 and A/H3N2 viruses. Continuous monitoring of genetic changes in the *NA* genes is critical to monitor the evolution and antivirus drug susceptibility of influenza viruses.

Author contributions All authors contributed extensively to the work presented in this paper.

Compliance with ethical standards

Conflict of interest The authors have no conflicts of interest to disclose.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the Pasteur's institutional research committee of Iran and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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