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ORIGINAL ARTICLE

Assessment of influenza A (H1N1, H3N2) oseltamivir resistance during 2017-2019 in Iran

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ABSTRACT

Background and Objectives: Neuraminidase inhibitors (NAIs) as an imperative antiviral for influenza prophylaxis and treatment are being consumed worldwide. Increasing use of these antivirals might be associated with drug resistance. Regarding the significance of these variations, this study aimed to investigate the mutations occurring in the NA gene of influenza A viruses leading to oseltamivir resistance during 2017-2019 in Iran.

Materials and Methods: In this cross-sectional study, 40 influenza A (H1N1, H3N2) strains, isolated in National Influenza Center (NIC) from patients with Severe Acute Respiratory Infection (SARI) during 2017-2019 were subjected to RT-PCR and sequencing of NA complete gene. The frequency of oseltamivir resistance and variation of NA amino acids in these strains were investigated.

Results: No significant mutation conferring oseltamivir resistance was detected. However, NA antigenic sites in these strains depicted minor changes compared to the vaccine strains. Among H3N2 isolates, mutations at 329, 344, 346 and 385 and among H1N1 isolates mutations at 143 and 188 residues occurred in NA antigenic regions.

Conclusion: Evaluation of NA gene sequences, showed no resistant viruses to oseltamivir. Given that the viruses in the present study were the last viruses circulating in Iran before COVID-19 pandemic, the results will be beneficial to have a worthy comparison with the strains circulating after the pandemic. Constant monitoring for the emergence of drug-resistant variants and antigenic changes are crucial for all countries.

Keywords: Influenza A viruses; Neuraminidase; Oseltamivir; Antiviral drug; Iran

INTRODUCTION

Influenza virus is a major pathogen associated with serious public health problem and acute respiratory tract infections (1). Two remarkable processes named antigenic drift and antigenic shift can cause genomic and subsequently antigenic variations in influenza viruses every year (2, 3). These features allow them to escape from the host immune system easily (4). Undoubtedly, preventive approaches such as vaccination have almost succeeded in lowering infection rates. Conversely, the efficacy of the vaccine against the circulating virus strain varies each year (5). In this case, the first step is to take antiviral medications because in addition to their therapeutic potential, they can be used as prophylaxis (4, 6). When it comes to therapeutic treatment approaches, the influenza virus is not an easy pathogen to target. Three class-

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es of antivirals are currently available for influenza treatment: the adamantanes or M2 inhibitors (amantadine and rimantadine), polymerase inhibitors (baloxavir, pimodivir, and favipiravir), and neuraminidase inhibitors (NAIs) (7). The first class of antiviral agents named adamantanes include amantadine and rimantadine are M2 channel blockers. Both of these antivirals are no longer recommended by Centers for Disease Control and Prevention (CDC) due to emergence of predominant resistant strains of influenza viruses. One example in the second class is baloxavir which blocks virus replication and can be a suitable alternative to NAIs in cases of resistance (8, 9). NAIs are another class of drugs used against influenza viruses. So far there are three FDA-approved (Food and Drug Administration) anti influenza NAIs named Oseltamivir, Zanamivir, and Peramivir. Oseltamivir, under the brand name Tamiflu, helps to minimize influenza symptoms and shortens the recovery time by hindering new viral particles from being released. Zanamivir or Relenza, as an inhaling drug and Peramivir with its trade name Rapivab as an intravenous infusion, can be used both for treatment and prophylaxis of seasonal influenza. All mentioned NAIs drugs bind to catalytic site of viral neuraminidase (NA), rendering the influenza virus unable to release from its host cell and infect the neighboring cells (10).

Having error prone viral RNA polymerase, influenza viruses can generate variants which in the long term may form a new subtype that is no longer sensitive to NAIs antivirals. Prior to 2007, there was no evidence of oseltamivir resistance in influenza viruses. During 2007-2008, human cases of oseltamivir-resistant seasonal influenza A/H1N1 viruses with amino acid substitution Histidine to Tyrosine (H275Y) of NA appeared gradually (11, 12). The H275Y substitution is the most common mutation conferring oseltamivir resistance in the N1 subtype of the influenza virus (12, 13). Resistant mutations are more common in high-risk groups especially immunodeficient patients, due to higher viral loads and prolonged viral shedding. Nonetheless, H275Y substitution in the NA active site, are likely to occur in patient without the underlying disease (14, 15).

In general, NAIs resistant mutant strains rarely have been reported in Iran (11, 16-18). However, some studies reported a limited number of resistant strains containing the most common substitution "H275Y" in influenza A/H1N1 particularly in high-risk individuals mainly in transplant recipients' elderlies and patients with underlying medical conditions (19). Nonetheless, NAIs are still drugs of choice to combat influenza A infections in Iran.

Among the most frequent amino acid substitutions, E119V and R292K, found predominantly in influenza A/H3N2, are associated with reduced susceptibility to oseltamivir (20). Even though, it is not clear how these mutations affect enzymatic activity.

Continuous monitoring of NA genetic changes is needed for risk assessment of NAIs resistance in influenza A viruses (21, 22). Given the emergence of drug resistance to adamantanes, certainly NAIs are of great importance in terms of treatment and prevention. Therefore, the possibility of developing resistant strains to NAIs should be considered (9, 23, 24).

Meanwhile, antibodies against NA are important in protection against influenza in humans (25, 26). Since NA is one of the targets for influenza vaccination, genetic changes should be continuously monitored for assessment of antigenic changes (27).

The present study aimed to evaluate the possible mutations that occur in NA genes of influenza A (H1N1, H3N2) viruses, leading to oseltamivir resistance and antigenic changes among isolated strains from respiratory clinical specimens of patients admitted to the hospital with Severe Acute Respiratory Infections (SARI) who referred to National Influenza Center (NIC) during 2017-2019.

MATERIALS AND METHODS

Sample collection. In this cross-sectional study, 40 influenza A H1N1, H3N2 strains, isolated in NIC laboratory from hospitalized patients with SARI during 2017-2019, were subjected to RT-PCR and sequencing.

RNA extraction. Total RNA extraction from the inoculated Madin-Darby Canine Kidney (MDCK) cell culture supernatant or amniotic and allantoic fluid of embryonic eggs by influenza A H1N1, H3N2 viruses were conducted, using the High Pure Viral Nucleic Acid Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions.

RT-PCR, sequencing, and analysis. RT-PCR was performed on all extracted RNAs using World Health Organization recommended primers (refer to Table

1) (28) to amplify whole NA gene in two fragments. Amplification was performed by one step RT-PCR technique using One-Step RT-PCR Kit (QIAGEN, Germany) in a standard 25-µl reaction mixture, as follow: A total of 2.5 µl of RNA was added to 22.5 µl of the reaction mixture containing 5 µl of 5× One Step RT-PCR buffer, 1 µl of dNTP Mix, 1 µl One Step RT-PCR Enzyme Mix, 1.25 µl of each forward and reverse primers at a final concentration of 10 pmol and 13 µl of RNase free water. Reverse transcription and amplification were performed by PeqLab thermocycler (Germany) under the following conditions: 1 cycle at 60°C for 1 min, 1 cycle at 50°C for 30 min and 1 cycle at 95°C for 15 min, followed by 40 cycles of amplification at 94°C for 30s, 57°C for 30s and 72°C for 1 min, and a final extension cycle of 72°C for 10 min (totally 150 min). Then the RT-PCR products were electrophoresed on a 1.5% agarose gel with TBE and visualized by safe stain.

The amplicon products were sequenced using the ABI BigDye® Terminator Cycle Sequencing Kit v3.1, on the 3130 Genetic Analyzer Automated Sequencer (Applied Biosystems Foster City, California, USA). The amplicon sequences were aligned and compared with vaccine strains by BioEdit (v 7.0.5.3) to assess amino acids variations and potential oseltamivir resistant mutations. Then, phylogenetic analysis was performed using MEGA X based on the Neighbor joining method and bootstrap analyses by 1000 resampling of the datasets.

RESULTS

In the current study, 40 influenza A strains including 31 (77%) A/H3N2 and 9 (33%) influenza A/H1N1, isolated from respiratory samples of patients with SARI in Iran NIC during 2017-2019, were evaluated to identify oseltamivir resistant mutants and antigenic variations in NA gene. To reach this goal, the presence of E119V, D151E, I222V, R224K, E276D, N249S, R292K, N329K, S331R and R371K mutations in influenza A/H3N2 virus strains and E119V, I222N, E229N and H275Y in influenza A/H1N1 strains were checked. The results showed, none of the mentioned mutations were found in this study. Besides, amino acid sequence analysis showed no changes in conserved residues of catalytic and framework sites of NA in all strains.

However, NA full genome sequencing revealed some amino acid substitutions both in N1 and N2 that had no impact on oseltamivir resistance, but they might have some effects on the antigenicity. (Tables 2-4) (29-31).

By analyzing 31 NA sequences of influenza A/H3N2 viruses, it was observed that amino acids at residues 245 and 247 have changed in 8 strains compared to the correspondence vaccine strains (Table 2). S245N and S247T substitutions introduce an N-linked glycosylation site.

Phylogenetic analysis. Phylogenetic analysis of NA sequence of H1N1 and H3N2 strains studied here were compared with those of reference strains and other circulating viruses around the world. The reference sequences and strains from other countries were obtained from NCBI.

The 2017–2019 Iranian strains are indicated in different colors (strains from 2017-2018 influenza season are shown in green, strains from influenza 2018-2019 season are shown in blue). In accordance with the branching of the phylogenetic trees (Figs. 1 and 2), the NA genes of Iranian influenza A/H1N1 strains during seasons 2017-2018 and 2018-2019 were 98.4% similar to their correspondence vaccine strain, A/Michigan/45/15. The average similarity to the cor-

Table 1. Two sets of used primers for N1 and N2 to amplify two overlapping fragments for each gene.

No	Oligo Name	Position (nt)	Sequence
1	PN1F1	1-21	ATG AAT CCA AAC CAA AAG ATA ATA AC
2	PN1R1	952-937	ACT GCA TAT GTA TCC TAT CTG
3	PN1F2	674-694	GAA CAC AAG AGT CTG AAT GTG
4	PN1R2	1406-1386	TTG TCA ATG GTA AAT GGC AAC
5	N2F1	1-24	AGC AAA AGC AGG AGT GAA AAT GAA
6	N2R1	1100-1077	ATC CAC ACG TCA TTT CCA TCG TCA
7	N2F2	383-406	CAT GCG ATC CTG ACA AGT GTT ATC
8	N2R2	1443-1420	TTC TAA AAT TGC GAA AGC TTA TAT

Table 2. Amino acid substitutions in NA of influenza A/H3N2 strains compared to the vaccine strain during 2017-18.

Virus strain	Amino acid positions														
	75	**93**	**126**	**148**	**220**	**231**	**245**	**247**	**267**	**303**	**329***	**339**	**380**	**392**	**468**
A/HONG KONG/4801/2014 (vaccine)	K	G	P	K	K	I	S	S	T	V	N	D	I	T	P
A/Tehran/78090/17	R	-	L	T	N	V	N	T	K	I	S	N	V	I	H
A/Tehran/77284/17	-	-	-	T	N	V	N	T	K	I	S	N	V	I	H
A/Lorestan/77953/17	R	-	L	T	N	V	N	T	K	I	S	N	V	I	H
A/Varamin/75924/17	-	-	-	T	-	V	N	T	K	-	-	N	V	I	H
A/Tehran/100089/17	-	-	-	T	N	V	N	T	K	I	-	N	V	I	H
A/Tehran/99538/17	-	D	-	T		V	N	T	K	-	-	N	V	I	H
A/Tehran/93702/17	-	D	-	T	N	V	N	T	K	I	S	N	V	I	H
A/Tehran/91529/17	-	-	-	T	N	V	N	T	K	I	S	N	V	I	H

* = NA antigenic site

Table 3. Amino acid substitutions in NA of influenza A/H3N2 strains compared to the vaccine strain during 2018-19.

Virus strain	Amino acid positions															
	77	**126**	**212**	**220**	**231**	**263**	**307**	**315**	**329***	**331**	**338**	**344***	**346***	**351**	**352**	**385***
A/SINGAPORE/INFIMH-16-0019/2016 (vaccine)	I	P	I	K	V	V	I	S	N	S	L	E	G	G	W	N
A/Tehran/167623/19	-	L	V	N	-	I	-	-	S	-	-	-	-	-	-	-
A/Lorestan/168206/19	V	L	V	N	I	-	M	-	S	-	-	K	D	-	-	-
A/Isfahan/168336/19	-	L	V	N	-	-	-	R	S	-	-	K	D	-	-	-
A/Tehran/168359/19	-	L	V	N	-	I	-	-	S	-	-	-	-	-	-	-
A/Iran/168555/19	-	L	V	N	-	-	-	R	S	-	-	K	D	-	-	-
A/Astara/171909/19	-	L	V	N	-	-	-	R	S	-	-	K	-	-	-	-
A/Tehran/154296/19	V	L	V	N	-	-	-	R	S	-	-	-	D	-	-	-
A/Karaj/153427/19	-	L	V	N	-	-	-	-	S	-	-	-	-	-	-	T
A/Tehran/154193/19	-		V	N	-	I	-	-	S	-	-	-	-	-	-	-
A/Karaj/153084/19	-	L	V	N	-	I	-	-	S	-	-	-	-	-	-	-
A/Zanjan/136946/18	-	L	V	N	-	I	-	-	S	-	-	-	-	A	-	T
A/Tehran/155411/19	-	L	V	N	-	-	-	-	S	T	F	-	-	-	-	-
A/Tehran/168072/19	-	L	V	N	I	-	M	-	S	T	-	-	-	-	L	-
A/Lorestan/151692/18	-	L	V	N	-	-	-	-	S	-	-	-	-	-	-	-
A/Tehran/151574/18	-	L	V	N	-	I	-	-	S	-	-	-	-	-	-	-

* = NA antigenic site

responding vaccine strain for influenza A/H3N2 viruses during seasons 2017-2018 and 2018-2019 were 97.1% and 98.4% respectively.

DISCUSSION

Mutations in influenza viruses, like the most of RNA viruses, occur frequently due to the lack of viral RNA polymerase proofreading. These variations create mutants that are no longer preventive by vaccine (32). Therefore, antiviral medications can be of great importance in prophylaxis until new vaccine is available. With increasing use of NAIs, the widespread concern on the probability of emergence of NAIs resistant strains is much higher than before. Basically, factors such as patient's age, medication history, presence or absence of underlying disease, vi-

Table 4. Amino acid substitutions in NA of influenza A/H1N1 strains compared to the vaccine strain during 2017-18, 2018-19

Virus strain	Amino Acid positions										
	28	51	74	77	81	143*	188*	227	314	421	454
A/Michigan/45/15 (vaccine)	X	Q	F	G	V	K	I	N	M	D	N
A/Hamedan/162976/19	N	K	S	R	A	-	T	-	-	N	D
A/Hamedan/164026/19	N	-	-	R	A	-	T	D	-	N	D
A/Tehran/96481/17	N	-	-	R	A	-	T	-	-	N	D
A/Tehran/137043/18	N	-	-	R	A	R	T	-	I	-	D
A/Tehran/137742/18	N	-	-	R	A	R	T	-	I	-	D
A/Tehran/138894/18	N	-	-	R	A	-	T	D	-	N	D

* = NA antigenic site

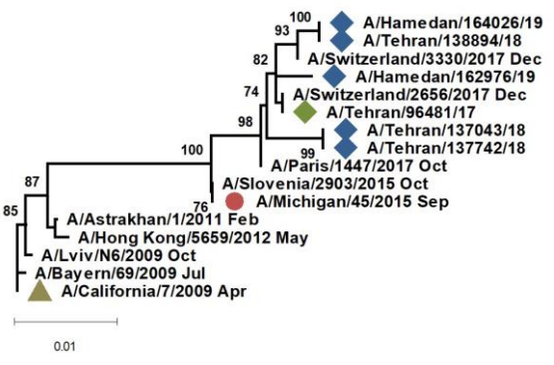

Fig. 1. Phylogenetic tree for NA drawn by neighbor-joining method with Tamura–Nei model MEGA-X software. The neuraminidase (NA) gene of nine A/H1N1 strains are indicated as follow: Iranian strains (◇), the vaccine strain (●) and strains from other countries and strain A/California/07/2009 as the root (▲). Bootstrap values based on 1000 replicates are shown at each main branch.

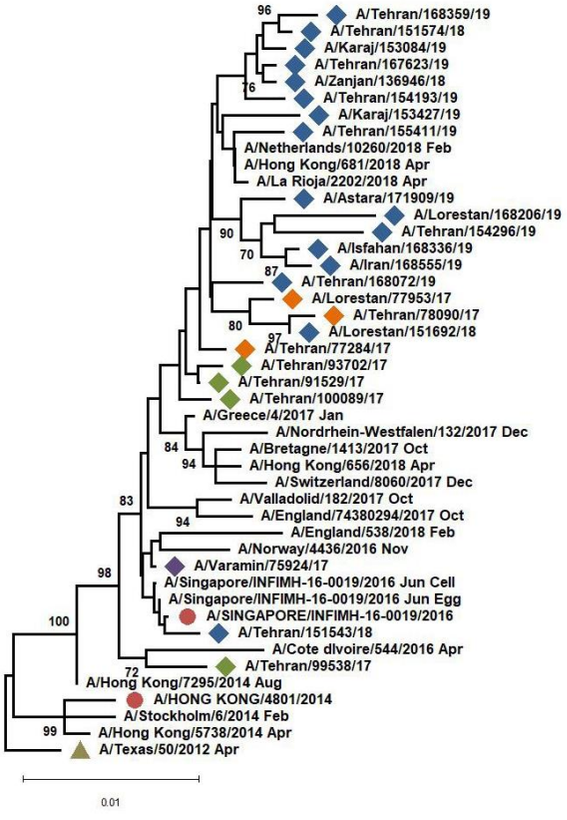

Fig. 2. Phylogenetic tree for NA drawn by neighbor-joining method with Tamura–Nei model MEGA-X software. The neuraminidase (NA) gene of 31 A/H3N2 strains are indicated as follow: Iranian strains (◇), the vaccine strain (●) and strains from other countries and strain A/Texas/50/2012 as the root (▲). Bootstrap values based on 1000 replicates are shown at each main branch.

rus type or subtype especially in immunosuppressed people, affect the results of studies on drug resistance of influenza viruses (15, 33). However, there is no report that resistance to oseltamivir could lead to cross-resistance with other NAIs. Therefore, patients with oseltamivir resistant influenza virus infection, can be easily treated with other NAIs (34, 35).

Currently, the NAIs resistance in Iran is low however with the outbreak of annual influenza epidemics and subsequently high consumption of NAIs antivirals, some resistant strains are expected to emerge. Under such circumstances, vaccines NAIs are not effective. Therefore, the reported mutations related to NAIs resistance in Iran can be remarkable in advancing treatment policies (11, 16, 17).

It should be noted that, this study had some limitations. There was no information available regarding patients' underlying diseases, their immune system status and medication assisted treatment. Based on our results, none of the previously known substitutions conferring resistance to oseltamivir were de-

tected in the influenza A/H3N2 strains circulated in patients with SARI during 2017 to 2019.

In line with our study, Yavarian et al. showed that NA genes of influenza A/H3N2 viruses circulating during 2005-2007 had none of mutations associated with the drug resistance (36). Moaser et al. investigated the properties of influenza virus NA gene of 35 A/H3N2 strains in 2010–2015. They reported that, among influenza A/H3N2 strains, no mutations associated with reduced susceptibility to NAIs were found (17). However, several studies have indicated the presence of drug-resistant mutations in NA. A study was conducted in Canada in 2011 on an immunosuppressed child who was treated with oseltamivir. They found "I222V" and E119V substitutions in NA gene conferring resistance to oseltamivir (37). Globally, oseltamivir resistance is rare and more frequently can be detected in children and immunocompromised individuals after prolonged drug exposure or sub therapeutical drug levels (3, 9). While some reports suggested that NAIs resistance can occur in the absence of oseltamivir exposure which indicates resistant mutants are able to maintain their replicative fitness and transmissibility (9, 18). Okomo-Adhiambo et al. indicated that NA gene sequences of influenza A/H3N2 viruses isolated from an immunocompromised patient had E119V and E119I substitutions. They suggested that detection of the mutant viruses might be limited to virus isolation in MDCK cells, where such virus variants had an apparent growth advantage over wild-type viruses (38). However, others pointed the role of new sequence-based assays like next-generation sequencing for detection of resistance markers in viruses in clinical specimens, prior to their isolation and propagation in cell culture (39, 40).

NA as the second most abundant glycoprotein on the virion surface induces specific antibodies which decrease viral load by interfering with the release of progeny viruses from the cell surface (35, 41). Every amino acid substitution occurring in the antigenic region of NA could be a prediction for the emergence of resistant mutant. Comparing influenza A (H1N1, H3N2) strains during 2017-2019 in Iran with the other studies sequences around the world showed some substitutions in the antigenic regions. Accordingly, we observed a number of amino acid mutations in antigenic sites which may reduce the effect of antibodies against previous vaccine strains. Among mutated antigenic sites, residues at positions 188 and 143 in the NA sequence of H1N1 were found. Interestingly, the same I188T mutation has been detected in a study conducted by Liu et al. (31). Sequence analysis of NA in H3N2 revealed a number of substitutions which were previously reported in association with antibody escape mutants or antibody binding affinity. Amino acid substitutions at positions 329, 344, 346 and 385 found in the current study, were in line with the other studies conducted by Kaplan et al. (30) and Liang et al. (29) proving that antibody binding to even two of these sites inhibits NA sialidase activity.

Among important mutations, substitution in sites of 245 and 247 were observed in 8 strains of influenza A/H3N2 viruses. The glycosylation at NA245 decreases the enzymatic activity of NA, also reduces the affinity of monoclonal antibodies. Previous studies showed that these substitutions introduced an N-linked glycosylation site and significantly decreased NA enzymatic activity by decreasing substrate access to the active site of the protein. Also they described the impact of this 245 N-linked glycosylation on antibody binding which caused alteration in antigenic properties (42-44).

The present study also investigated the NA sequence of 9 influenza A/H1N1 viruses circulated in patients with SARI during 2017 to 2019. None of the previously known substitutions conferring resistance to oseltamivir were detected in these strains. The results were in agreement with those obtained in the previous studies in neighboring region of Iran. For instance, in Pakistan in 2009-2010 among 14 A/H1N1 strains, none of them had resistance mutations to NAIs (45). Similarly, Shafiei et al. showed that influenza A/H1N1 isolates in Iran had no H275Y mutation (46). Also in a study that investigated the genetic characteristics of NA in Middle East and North Africa from 2009 to 2017, among 20725 A/H3N2 strains none of them had NAIs resistance-related mutations (47). In a survey conducted during 2009-2013 in Iran nucleotide similarity of H1N1 NA compared to the vaccine strains of the relevant seasons were 99.41%, 98.60% and 98.07%. Based on this information, the average similarity of NA in comparison with seasonal corresponding vaccine strain indicates that NA sequence does not drastically change over time (16). However, some changes were detected in NA antigenic sites. It should be noted that, antigenic changes in influenza viruses' surface glycoproteins (either HA or NA) could result in vaccine ineffectiveness and requiring influenza vaccine reformulation (48). This reformulation was happened both for influenza

virus H1N1 and H3N2 subtypes in 2019-2020 influenza season due to HA and NA antigenic variations. Herein, phylogenetic analysis of NA genes of Iranian influenza A (H1N1 and H3N2) strains showed they were similar to the correspondence vaccine strains (Figs. 1 and 2).

Despite the limited reports of NAIs resistance mutants globally, the increasing use of NAIs and probable emergence of NAIs resistance in influenza viruses highlights the need for drug resistance evaluation (16). Meanwhile, early treatment initiation and the appropriate dose and combination antiviral chemotherapy may minimize the likelihood of arising resistant viruses. Periodic evaluation of genomic sequences of influenza A viruses and annual monitoring of drug resistant influenza A/H1N1 and A/H3N2 viruses can be of tremendous value in limiting the emergence of resistant strains.

CONCLUSION

According to the objectives of this study, evaluation of NA gene sequence in influenza A/H1N1 and A/H3N2 circulating during 2017-2019 and comparison with correspondence vaccine strains showed no oseltamivir resistant mutant including E119V, D151E, I222V, R224K, E276D, N249S, R292K, N329K, S331R and R371K substitutions in influenza A/H3N2 strains and E119V, I222R/V, S247N, H275Y, N294S mutations in influenza A/H1N1 viruses. As, information on the prevalence of the resistance mutant of H1N1 and H3N2 influenza viruses in Iran is very limited, the continuous molecular monitoring of NA gene of influenza A viruses for effective management of treatment strategies is essential.

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