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-
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’ elderly 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
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-

### RESULTS

In the current study, 40 influenza A strains including 31 (77%) A/H3N2 and 9 (23%) influenza A/H1N1, isolated from respiratory samples of patients with SARI in Iran NIC during 2017-2019, were evaluated

| 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 CAT 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 AC GTC TTT CCA TCG TCA |
| 7  | N2F2       | 383-406       | CAT GGC ATC CTG ACA AGT GTT ATC |
| 8  | N2R2       | 1443-1420     | TTC TAA AAT TGC GAA AGC TTA TAT |

Table 1. Two sets of used primers for N1 and N2 to amplify two overlapping fragments for each gene.
**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 93 126 148 220 231 245 267 303 329* 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 - - - - - - |
| 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 - T |
| 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   | 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.

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-
ected 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 “I22V” 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-Adhiambbo 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 corresponding vaccine strains (Figs. 1 and 2).

Despite the limited reports of NAs resistance mutants globally, the increasing use of NAs and probable emergence of NAs 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, I222Y, 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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REFERENCES

1. Shatizadeh S, Yavarian J, Rezaie F, Mahmoodi M, Nasiri M, Mokhtari Azad T. Epidemiological and clinical evaluation of children with respiratory virus infections. Med J Islam Repub Iran 2014; 28: 102.
2. Souquette A, Thomas PG. Past life and future effects-How heterologous infections alter immunity to influenza viruses. Front Immunol 2018; 9: 1071.
3. Tavakoli F, Moattari A, Shamsi Shahr Abadi M, Kadivar MR, Khodadad N, Pirbonyeh N, et al. Antigenic Variation of the Haemagglutinin Gene of the Influenza A (H1NI) pdm09 Virus Circulating in Shiraz, February-April 2013. Iran J Immunol 2015; 12: 198-208.
4. Jackson ML, Chung JR, Jackson LA, Phillips CH, Benoit J, Monto AS, et al. Influenza vaccine effectiveness in the United States during the 2015–2016 season. N Engl J Med 2017; 377: 534-543.
5. Bassetti M, Castaldo N, Carnelutti A. Neuraminidase inhibitors as a strategy for influenza treatment: pros, cons and future perspectives. Expert Opin Pharmacother 2019; 20: 1711-1718.
6. Paules CI, Sullivan SG, Subbarao K, Fauci AS. Chasing seasonal influenza-the need for a universal influenza vaccine. N Engl J Med 2018; 378: 7-9.
7. Sautto GA, Kirchenbaum GA, Ross TM. Towards a universal influenza vaccine; different approaches for one goal. Virol J 2018; 15: 17.
8. Hayden FG, Sugaya N, Hirotsu N, Lee N, de Jong MD, Hurt AC, et al. Baloxavir marboxil for uncomplicated influenza in adults and adolescents. N Engl J Med 2018; 379: 913-923.
9. Heo Y-A. Baloxavir: first global approval. Drugs 2018; 78: 693-697.
10. Han N, Oh JM, Kim I-W. Assessment of adverse events related to anti-influenza neuraminidase inhibitors using the FDA adverse event reporting system and online patient reviews. Sci Rep 2020; 10: 3116.
11. Rashidi O, Moattari A, Pirbonyeh N, Emami A, Kadivar MR, Tavakoli Movaghar N, et al. Investigation of genetic variation: Neuraminidase gene of influenza A virus H1NI/pdm09, Shiraz, Iran (2015–2016). J Med Virol 2021; 93: 4763-4772.
12. Holmes EC, Hurt AC, Dobbie Z, Clinch B, Oxford JS, Piedra PA. Understanding the impact of resistance to influenza antivirals. Clin Microbiol Rev 2021; 34(2): e00224-20.
13. Zhang W, Xu H, Guan S, Wang C, Dong G. Frequency and distribution of H1N1 influenza A viruses with oseltamivir-resistant mutations worldwide before and after the 2009 pandemic. J Med Virol 2022; 94: 4406-4416.
14. Liu S-S, Jiao X-Y, Wang S, Su W-Z, Jiang L-Z, Zhang X, et al. Susceptibility of influenza A (H1Ni)/pdm2009, seasonal A (H3N2) and B viruses to Oseltamivir in Guangdong, China between 2009 and 2014. Sci Rep 2017; 7: 8488.
15. Lampejo T. Influenza and antiviral resistance: an overview. Eur J Clin Microbiol Infect Dis 2020; 39: 1201-
16. Momeni P, Abedin Dargoosh S, Sedehzadeh AA, Bagheri G, Mohammadi M, Pooshashkan L, et al. Neuraminidase Gene Variations in Influenza A (H1N1) pdm09 Virus among Patients Admitted to Referral Pulmonary Hospital, Tehran, Iran in 2009–2013. *Tianaffos* 2017; 16: 99-106.

17. Moasser E, Behzadian F, Moattari A, Fotouhi F, Zarate H. Characterization of the neuraminidase genes from human influenza A viruses circulating in Iran from 2010 to 2015. *Arch Virol* 2018; 163: 391-400.

18. Khodadad N, Moattari A, Shamsi Shahr Abadi M, Kadivar MR, Sarvari J, Tavakoli F, et al. Prevalence of influenza A (H1N1) pdm09 virus resistant to oseltamivir in Shiraz, Iran, during 2012-2013. *Jandishapour J Microbiol* 2015; 8(8c): e23690.

19. Moradi A, Nadji SA, Tabarsi P, Hashemian SM, Marjani M, Sigaroodi A, et al. Prevalence of oseltamivir-resistant 2009 H1N1 influenza virus among patients with pandemic 2009 H1N1 influenza infection in NRITLD, Tehran, Iran. *Tianaffos* 2011; 10: 8-11.

20. Koel BF, Vigeveno RM, Pater M, Koekkoek SM, Han AX, Tian HM, et al. Longitudinal sampling is required to maximize detection of intrahost A/H3N2 virus variants. *Virus Evol* 2020; 6:vnea088.

21. Takashita E, Daniels RS, Fujisaki S, Gregory V, Gubareva LV, Huang W, et al. Global update on the susceptibility of human influenza viruses to neuraminidase inhibitors and the cap-dependent endonuclease inhibitor baloxavir, 2017–2018. *Antiviral Res* 2020; 175: 104718.

22. Akand EH, Downard KM. Mechanisms of antiviral resistance in influenza neuraminidase revealed by a mass spectrometry based phylonomics approach. *Mol Phylogenet Evol* 2019; 135: 286-296.

23. Pires De Mello CP, Drusano GL, Adams JR, Shudt M, Kulawy R, Brown AN. Oseltamivir-zanamivir combination therapy suppresses drug-resistant H1N1 influenza A viruses in the hollow fiber infection model (HFIM) system. *Eur J Pharm Sci* 2018; 111: 443-449.

24. Ishiguro N, Koseki N, Kaiho M, Ariga T, Kikuta H, Oba K, et al. Clinical effectiveness of four neuraminidase inhibitors (oseltamivir, zanamivir, lanaminivir, and peramivir) for children with influenza A and B in the 2014–2015 to 2016–2017 influenza seasons in Japan. *J Infect Chemother* 2018; 24: 449-457.

25. Job ER, Schotsaert M, Ibañez LI, Smet A, Ysenbaert T, Roose K, et al. Antibodies directed toward neuraminidase N1 control disease in a mouse model of influenza. *J Virol* 2018; 92(4): e01584-17.

26. Sedova ES, Scherinbin DN, Lysenko AA, Alekseeva SV, Artemova EA, Shmarov MM. Non-neutralizing antibodies directed at conservative influenza antigens. *Acta Naturae* 2019; 11: 22-32.

27. McMillan CLD, Young PR, Watterson D, Chappell KJ. The next generation of influenza vaccines: Towards a universal solution. *Vaccines (Basel)* 2021; 9: 26.

28. Yavarian J, Shafiei Jandaghi NZ, Naseri M, Mokhtari Azad T. Characterization of variations in PB2, NS1, M, neuraminidase and hemagglutinin of influenza A (H3N2) viruses in Iran. *Jandishapour J Microbiol* 2014; 7(3): e9089.

29. Liang L, Huang P, Wen M, Ni H, Tan S, Zhang Y, et al. Epitope peptides of influenza H3N2 virus neuraminidase gene designed by immunoinformatics. *Acta Biochim Biophys Sin (Shanghai)* 2012; 44: 113-118.

30. Kaplan BS, Anderson TK, Chang J, Santos J, Perez D, Lewis N, et al. Evolution and antigenic advancement of N2 neuraminidase of swine influenza A viruses circulating in the United States following two separate introductions from human seasonal viruses. *J Virol* 2021; 95(20): e0063221.

31. Liu B, Wang Y, Liu Y, Chen Y, Liu Y, Cong X, et al. Molecular evolution and characterization of hemagglutinin and neuraminidase of influenza A (H1N1) pdm09 viruses isolated in Beijing, China, during the 2017–2018 and 2018–2019 influenza seasons. *Arch Virol* 2021; 166: 179-189.

32. Mohan T, Nguyen HT, Kniss K, Mishin VP, Merced-Morales AA, Laplante J, et al. Cluster of Oseltamivir-Resistant and Hemagglutinin Antigenically Drifted Influenza A (H1N1) pdm09 Viruses, Texas, USA, January 2020. *Emerg Infect Dis* 2021; 27: 1953-1957.

33. Lee N, Hurt AC. Neuraminidase inhibitor resistance in influenza: a clinical perspective. *Curr Opin Infect Dis* 2018; 31: 520-526.

34. Principi N, Camilloni B, Alunno A, Polinori I, Argen-tiero A, Esposito S. Drugs for influenza treatment: is there significant news? *Front Med (Lausanne)* 2019; 6: 109.

35. Bai Y, Jones JC, Wong S-S, Zanin M. Antivirals targeting the surface glycoproteins of Influenza virus: Mechanisms of action and resistance. *Viruses* 2021; 13: 624.

36. Yavarian J, Mokhtari-Azad T, Nadji SA, Zeraati H, Naseri M. Analysis of the hemagglutinin and neuraminidase genes of human Influenza A/H3N2 viruses circulating in Iran between 2005 and 2007: antigenic and phylogenetic relationships to vaccine strains. *Intervirology* 2010; 53: 133-140.

37. Simon P, Holder BP, Bouhy X, Abed Y, Beauchemin CA, Boivin G. The I222V neuraminidase mutation has a compensatory role in replication of an oseltamivir-resistant influenza virus A/H3N2 E119V mutant. *J Clin Microbiol* 2011; 49: 715-717.

38. Okomo-Adhiambo M, Demmler-Harrison GJ, Dey-de VM, Shue TG, Xu X, Klimov AI, et al. Detection of E119V and E119I mutations in influenza A (H3N2) viruses isolated from an immunocompromised pa-
tient: challenges in diagnosis of oseltamivir resistance. Antimicrob Agents Chemother 2010; 54: 1834-1841.
39. Van Poelvoorde LAE, Saelens X, Thomas J, Roosens NH. Next-generation sequencing: an eye-opener for the surveillance of antiviral resistance in influenza. Trends Biotechnol 2020; 38: 360-367.
40. McGinnis J, Laplante J, Shudt M, George KS. Next generation sequencing for whole genome analysis and surveillance of influenza A viruses. J Clin Virol 2016; 79: 44-50.
41. McAuley JL, Gilbertson BP, Trifkovic S, Brown LE, McKimm-Breschkin JL. Influenza virus neuraminidase structure and functions. Front Microbiol 2019; 10: 39.
42. Powell H, Pekosz A. Neuraminidase antigenic drift of H3N2 clade 3c. 2a viruses alters virus replication, enzymatic activity and inhibitory antibody binding. PLoS Pathog 2020; 16(6): e1008411.
43. Wan H, Gao J, Yang H, Yang S, Harvey R, Chen Y-Q, et al. The neuraminidase of A (H3N2) influenza viruses circulating since 2016 is antigenically distinct from the A/Hong Kong/4801/2014 vaccine strain. Nat Microbiol 2019; 4: 2216-2225.
44. Zost SJ, Parkhouse K, Gumina ME, Kim K, Diaz Perez S, Wilson PC, et al. Contemporary H3N2 influenza viruses have a glycosylation site that alters binding of antibodies elicited by egg-adapted vaccine strains. Proc Natl Acad Sci U S A 2017; 114: 12578-12583.
45. Bashir Aamir U, Badar N, Mehmood MR, Nisar N, Suleman RM, Shaukat S, et al. Molecular epidemiology of influenza A (H1N1) pdm09 viruses from Pakistan in 2009–2010. PLoS One 2012; 7(8): e41866.
46. Jandaghi NZ, Azad TM, Naseri M, Yavarian J, Nategh R. Molecular and genetic characteristics of hemagglutinin and neuraminidase in Iranian 2009 pandemic influenza A (H1N1) viruses. Arch Virol 2010; 155: 717-721.
47. Al Khatib HA, Al Thani AA, Gallouzi I, Yassine HM. Epidemiological and genetic characterization of pH1N1 and H3N2 influenza viruses circulated in MENA region during 2009–2017. BMC Infect Dis 2019; 19: 314.
48. Isakova-Sivak I, Stepanova E, Mezhenskaya D, Matyushenko V, Prokopenko P, Sychev I, et al. Influenza vaccine: Progress in a vaccine that elicits a broad immune response. Expert Rev Vaccines 2021; 20: 1097-1112.