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# Characterization of Human Influenza Viruses in Lebanon during 2010–2011 and 2011–2012 Post-Pandemic Seasons

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**Key Words**

Influenza virus · Pandemic H1N1 · Neuraminidase inhibitors · Antiviral drug susceptibility · Lebanon · Middle East

**Abstract**

***Objective:*** To genetically characterize human influenza viruses and their susceptibilities to antivirals during two post-pandemic seasons in Lebanon. ***Methods:*** Influenza virus was isolated from nasopharyngeal swabs that were obtained from patients with influenza-like illness during 2010–2012 and further analyzed both phenotypically and genotypically. ***Results:*** During the 2010–2011 season, both 2009 pandemic H1N1 (H1N1p) and B viruses co-circulated with equal prevalence, while the H3N2 virus predominated during the 2011–2012 season. All H3N2 and H1N1 viruses were resistant to amantadine. Importantly, all viruses of the influenza A and B types were susceptible to the neuraminidase (NA) inhibitors oseltamivir, zanamivir, peramivir, and laninamivir. Nonetheless, all 2011–2012 H1N1p isolates had three mutations (V241I, N369K, and N386S) in the NA gene that were suggested to be permissive of the H275Y mutation, which confers resistance to oseltamivir. We also detected one H1N1p virus during the 2010–2011 season with a 4-fold decrease in susceptibility to oseltamivir due to an NA-S247N mutation. This isolate was phylogenetically distinct from other H1N1p viruses that were isolated in other regions. ***Conclusions:*** Influenza A viruses with reduced susceptibility to oseltamivir and mutations permissive for acquiring NA resistance-conferring mutation with minimal burden on their fitness were isolated in Lebanon.

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## Introduction

Annual influenza outbreaks affect on average 5–20% of the world's population, resulting in a significant burden on human health [1]. These outbreaks are caused by seasonal influenza viruses that undergo continuous antigenic changes [2]. Occasionally, pandemics can occur due to antigenic shifts in the HA protein. Until the 2008–2009 winter season, two influenza A subtypes, H3N2 and H1N1, and influenza B circulated in humans. However, in spring 2009, a novel influenza virus (pandemic H1N1 2009; H1N1p) emerged and quickly spread around the

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world [3]. In the following season, the seasonal H1N1 virus continued to circulate at a minimal level but eventually became extinct. However, a return and, in some cases, dominance of seasonal H3N2 and influenza B viruses was observed worldwide [4, 5].

In addition to their antigenic evolution, influenza viruses evolve under drug pressure or natural selection, and new virus populations that are resistant to treatment occasionally evolve and sometimes prevail if viral fitness is maintained [6, 7]. As a result, the use of antiviral drugs like amantadine, an M2-channel inhibitor, has been largely limited due to the surge of amantadine-resistant H3N2 influenza viruses [8]. Neuraminidase inhibitors (NAIs), including oseltamivir and zanamivir, became the drugs of choice for treatment of H3N2 influenza infections [9]. The utility of oseltamivir in treating influenza was jeopardized by the worldwide rapid emergence of oseltamivir-resistant seasonal H1N1 viruses during 2007 through 2009 [6, 7]. Nonetheless, this virus was soon replaced by the H1N1p virus, which is largely sensitive to oseltamivir but resistant to amantadine [10]. This has revived the utility of oseltamivir in treating influenza.

Surveillance studies aimed at investigating the evolution and characteristics of influenza viruses worldwide are essential to early detection of new antigenically variant viruses as well as those with decreased antiviral drug susceptibility to allow informed recommendations for vaccination strategies and therapeutic interventions. Influenza surveillance efforts in the Middle East, including Lebanon, the site of this study, have been very limited. Characterization of influenza viruses in this region is critical to compliment the global effort to monitor and control influenza. The H1N1p influenza virus was first detected in Lebanon in May 2009. One study showed that a large outbreak caused by the pandemic virus occurred in October of the same year in Lebanon [11]. The prevalence of H1N1p viruses in the post-pandemic seasons and their susceptibilities to antiviral drugs is unknown in Lebanon.

The goal of this study was to characterize influenza viruses that circulated in Lebanon during two post-pandemic influenza seasons.

## Materials and Methods

*Sample Collection*

Nasal swabs were collected from patients presenting with influenza-like illness (ILI) symptoms (i.e. fever of at least 37.5°, cough, rhinorrhea, myalgia, and headache) at a pediatric clinic in Beirut. One swab was immediately tested using a rapid influenza diagnostic test, Quick-Ex Flu kit (Denka Seiken, Japan), and a second swab was stored in virus transport media at –80° until further analysis.

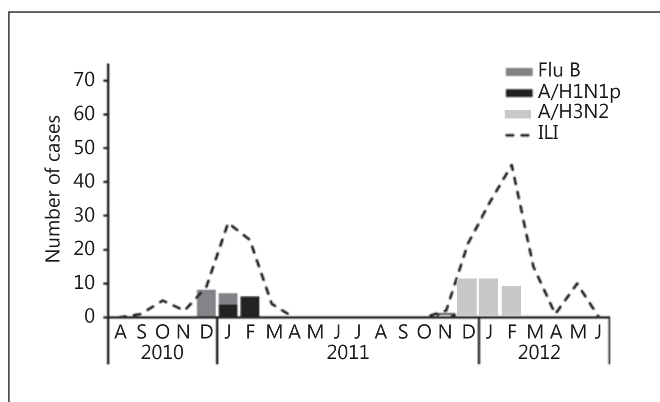


**Fig. 1.** Graphical representation of influenza cases in Lebanon during the 2010–2012 period. The dotted line represents all ILI cases that were identified during the study period. The letters on the x-axis represent months of the year.

*Virus Isolation and Characterization*

100 μl of the swab suspension was inoculated onto Madin-Darby canine kidney (MDCK) cells and incubated at 37° and 5% $CO_2$. Influenza virus type and subtype were characterized using cycling-probe real-time PCR [12–14].

*RNA Extraction, PCR, and Sequencing*

Following RNA extraction and reverse transcription [15, 16], the HA (HA1 subunit) and neuraminidase (NA) genes were sequenced and aligned using BioEdit software. Phylogenetic trees were constructed using the Neighbor-Joining (MEGA program). Clusters were defined using WHO-designated reference strains [17]. Sequences generated in this study were deposited in the Japanese GenBank database (http://www.ddbj.nig.ac.jp/; accession No. KF000008-62).

*Antiviral Drug Susceptibility Testing*

Genotypic analysis of the single-nucleotide polymorphisms M2-S31N and NA-H275Y, which confer resistance to amantadine and oseltamivir, respectively, was performed using cycling probe method [12, 13]. In vitro antiviral drug susceptibilities of the isolated viruses were determined by measuring the 50% inhibitory concentrations ($IC_{50}$) of oseltamivir (Sequoia Research, UK), zanamivir (Sequoia Research, UK), peramivir (Shionogi, Japan), and laninamivir (Daiichi Sankyo, Japan) using a fluorescence-based NA inhibition assay with methylumbelliferone N-acetylneuraminic acid (MUNANA) as the substrate at a final concentration of 0.025 mM [18].

## Results

A total of 202 samples were collected from patients with ILI during August 2010 through June 2012 (fig. 1). These included 73 samples from the 2010–2011 season and 129 samples from the 2011–2012 season. ILI activity

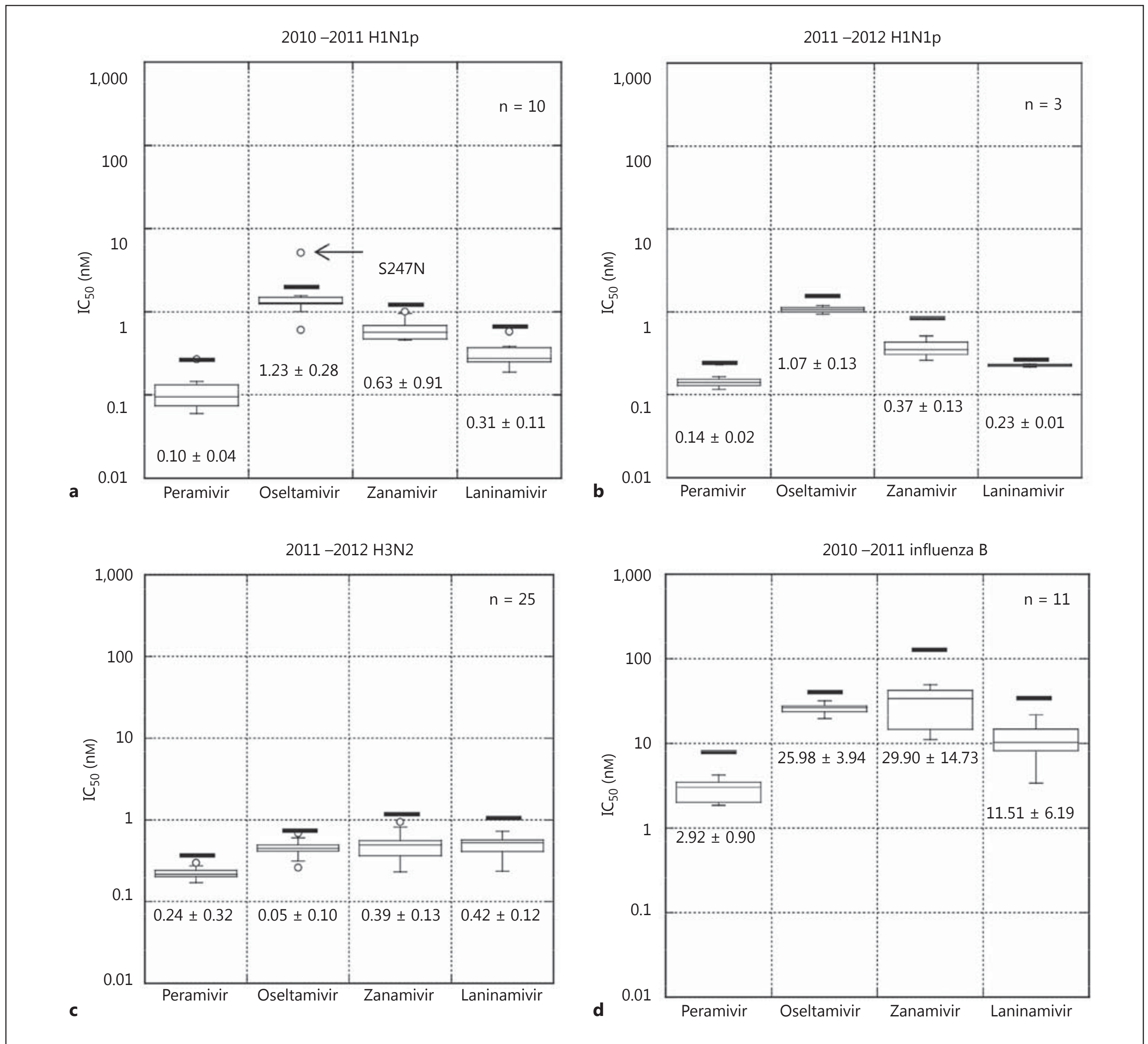


**Fig. 2.** Box plots of $IC_{50}$ values of NAIs against 2010–2011 H1N1p (**a**), 2011–2012 H1N1p (**b**), 2011–2012 H3N2 (**c**), and 2010–2011 influenza B (**d**) viruses collected in Lebanon. $IC_{50}$ values of peramivir, oseltamivir, zanamivir, and laninamivir were determined using a fluorescence-based assay with MUNANA as a substrate. The black bars indicate the 75th percentile + 3 × interquartile range (3 IQR). Values below the box plot represent mean ± SD. The mean value was calculated from viruses with $IC_{50}$ values below the cutoff limit (75th percentile + 3 IQR). Samples with $IC_{50}$ greater than the cutoff limit are considered outliers.

was highest during the months of January and February. In the 2010–2011 season, 12 (17%) samples tested positive for influenza A and 15 (21%) samples tested positive for influenza B using the rapid test kit. Among these, 10 H1N1p viruses and 11 influenza B viruses were isolated. The influenza B cases were detected in the earlier part of the season, followed by H1N1p viruses. No H3N2 viruses were detected during the 2010–2011 season. In the 2011–2012 season, 42 (33%) samples tested positive for influenza A by the rapid kit, and no influenza B viruses were

detected. Of these, 23 H3N2 and 3 H1N1p viruses were isolated.

Cycling probe real-time PCR analysis revealed that all viruses from both subtypes had the M2-S31N mutation. None of the H1N1p viruses from either the 2010–2011 or 2011–2012 season had the NA-H275Y mutation. Using an in vitro fluorescence-based NA inhibition assay, all H1N1p viruses collected in this study were found susceptible to all the tested NAIs with $IC_{50}$ values of 0.06–0.19 nM for peramivir, 0.6–5.11 nM for oseltamivir, 0.46–1.01 nM for zanamivir, and 0.19–0.57 nM for laninamivir (fig. 2a, b). One H1N1p isolate (A/Lebanon/10L-21/2011) had a 4-fold higher oseltamivir $IC_{50}$ ($IC_{50}$ = 5.11 nM) than the average $IC_{50}$ (1.18 nM) for the rest of the H1N1p isolates from both seasons. No difference was observed in $IC_{50values}$ of the other three NAIs against the 10L-21 isolate. For the H3N2 viruses, $IC_{50}$ values were 0.17–0.30 nM for peramivir, 0.94–1.2 nM for oseltamivir, 0.26–0.51 nM for zanamivir, and 0.23–0.73 nM for laninamivir (fig. 2c). Influenza B isolates had $IC_{50}$ values that were higher than the influenza A viruses at 1.85–4.27 nM for peramivir, 19.58–32.05 nM for oseltamivir, 11.10–49.18 nM for zanamivir, and 3.41–21.82 nM for laninamivir (fig. 2d). In summary, the $IC_{50}$ values suggest that all influenza A viruses isolated in the study are highly susceptible to NAIs. Influenza B isolates had up to 50-fold higher $IC_{50}$ values than influenza A isolates.

Phylogenetic analysis of the HA genes from H1N1p viruses revealed that the 2010–2011 season belonged to five distinct clusters: 1, 5, 6, 7 and 9 (fig. 3a). Cluster 1 included isolates from the 2009 pandemic outbreak with the exception of one isolate (A/Lebanon/10L-21/2011) from the 2010–2011 season, which had 2 additional mutations (S134T and R265K) in the HA gene. Two isolates (A/Lebanon/10L-34/2011 and A/Lebanon/10L-38/2011) from the 2010–2011 season formed a small cluster, designated cluster 9, characterized by 4 unique mutations (N44D, A267V, G270S, and I272L). None of these mutations belonged to any of the reported antigenic sites. In addition to the 2010–2011 season isolates, cluster 6 also accommodated the 2011–2012 isolates, which accumulated 3 additional HA mutations, including an H144R mutation in the Ca antigenic site and a V158I mutation adjacent to the Sa antigenic site [19]. Overall, the H1N1p viruses analyzed in this study had 6 amino acid mutations in the antigenic sites: 3 in Ca, 2 in Sb, and 1 in Sa, compared with A/California/07/2009. Three of these mutations were not detected in the earlier H1N1p viruses.

To determine the genetic basis of the reduced susceptibility to oseltamivir of the 10L-21 (A/Lebanon/10L-21/2011) isolate from the 2010–2011 season and its genetic relatedness to other isolates, we sequenced the NA genes of all H1N1p viruses isolated in this study. In general, the Lebanese H1N1p NA genes had a clustering pattern that corresponded with their counterpart HA clusters and had 5–7 mutations compared with the A/California/07/2009 vaccine strain (fig. 3). The 10L-21 isolate with reduced susceptibility to oseltamivir belonged to cluster 1 in the NA tree similar to its position in the HA tree. This isolate was characterized by 3 mutations: S247N, N385T, and N386K. The N385T and N386K mutations were located away from the enzyme-active site, suggesting no role in the reduced susceptibility to oseltamivir. The S247N mutation interacts with amino acid residues that in turn interact with oseltamivir and could interfere with stability of the inhibitor in the enzyme-active pocket [20]. The 10L-34 and 10L-38 strains formed a small isolated cluster in the NA tree just like in the HA tree and were characterized by 4 unique mutations: Q313E, T383R, I396T, and S442I.

The HA genes of 8 Lebanese H3N2 influenza viruses collected during the 2011–2012 season were sequenced. All the sequenced viruses were A/Perth/16/2009-like and belonged to the Vic/208 lineage in the HA phylogeny (fig. 4). Two of the isolates belonged to clade 3A, and the rest belonged to clade 3B, which also contained strains from other Middle Eastern countries. These isolates accumulated 9–10 mutations, including 2 antigenic site A mutations (K144N/D and N145S). In case of influenza B, all of the 11 isolates collected during the 2010–2011 season were sequenced. All of these isolates were Victoria-like and belonged to the Brisbane/60 clade (fig. 5). The Lebanese strains had 1–2 mutations compared with the B/Brisbane/60/2008 vaccine strain (2009–2012).

## Discussion

In this study, we found that in the first post-pandemic season (2010–2011), both influenza B and A/H1N1p cocirculated at a similar rate. In the 2011–2012 season, the A/H3N2 virus dominated and the H1N1p virus co-circulated with a lower prevalence. Similar circulation patterns have been reported elsewhere in the world [4, 21]. The extinction of seasonal H1N1 viruses has been suggested to be a consequence of the original antigenic sin, whereby infection with the H1N1p virus boosted the HA stalk antibodies against the seasonal H1N1 virus [22]. A similar phenomenon has been observed when H2N2 pandemic viruses, which emerged in 1957, replaced previously circulating H1N1 viruses. The H2N2 virus circulated for 11



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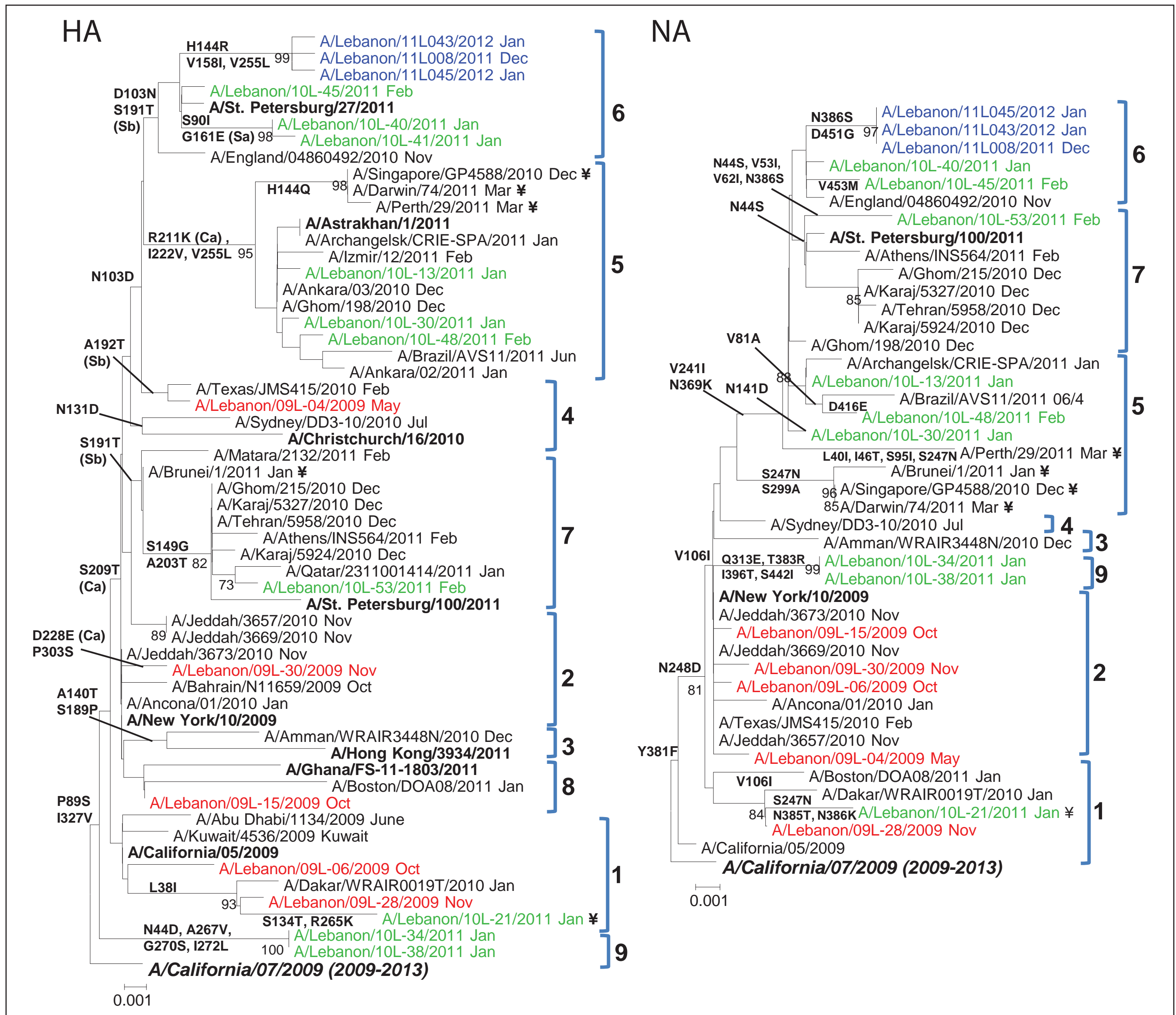


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**Fig. 3.** Phylogenetic analysis of the hemagglutinin (HA1) and NA genes of influenza A/H1N1 pandemic 2009 viruses isolated in Lebanon. The trees were constructed based on the nucleotide sequences using the Neighbor-Joining method with bootstrap analysis of 1,000 replicates. A/California/07/09 was used as the root for the tree and bootstrap values greater than 70% are shown. Amino acid mutations characteristic of the main clusters or the Lebanese isolates are indicated on the branches. The HA protein antigenic site to which a mutation belongs is in parentheses. Vaccine strains are in bold italics and reference strains are in bold. Lebanese isolates from 2009 (previously reported by Zaraket et al. [11]), 2010–2011, and 2011–2012 seasons are shown in red, green, and blue, respectively. ¥ Viruses with the S247N mutation. In the NA tree, only reference samples for which the NA gene sequence was available in the GenBank database are included.

years until it was replaced by H3N2 virus in 1968 due to antibodies specific to the retained N2 subtype [23].

The H1N1p viruses continued to evolve, accumulating mutations in antigenic sites, but remained antigenically similar to the A/California/07/2009 vaccine strain. In the post-pandemic 2010–2011 season, H1N1p viruses collected in Lebanon were diverse, belonging to multiple clusters, suggesting multiple introductions of this virus into Lebanon. This was in contrast to mostly homologous H1N1p viruses that we detected in previous

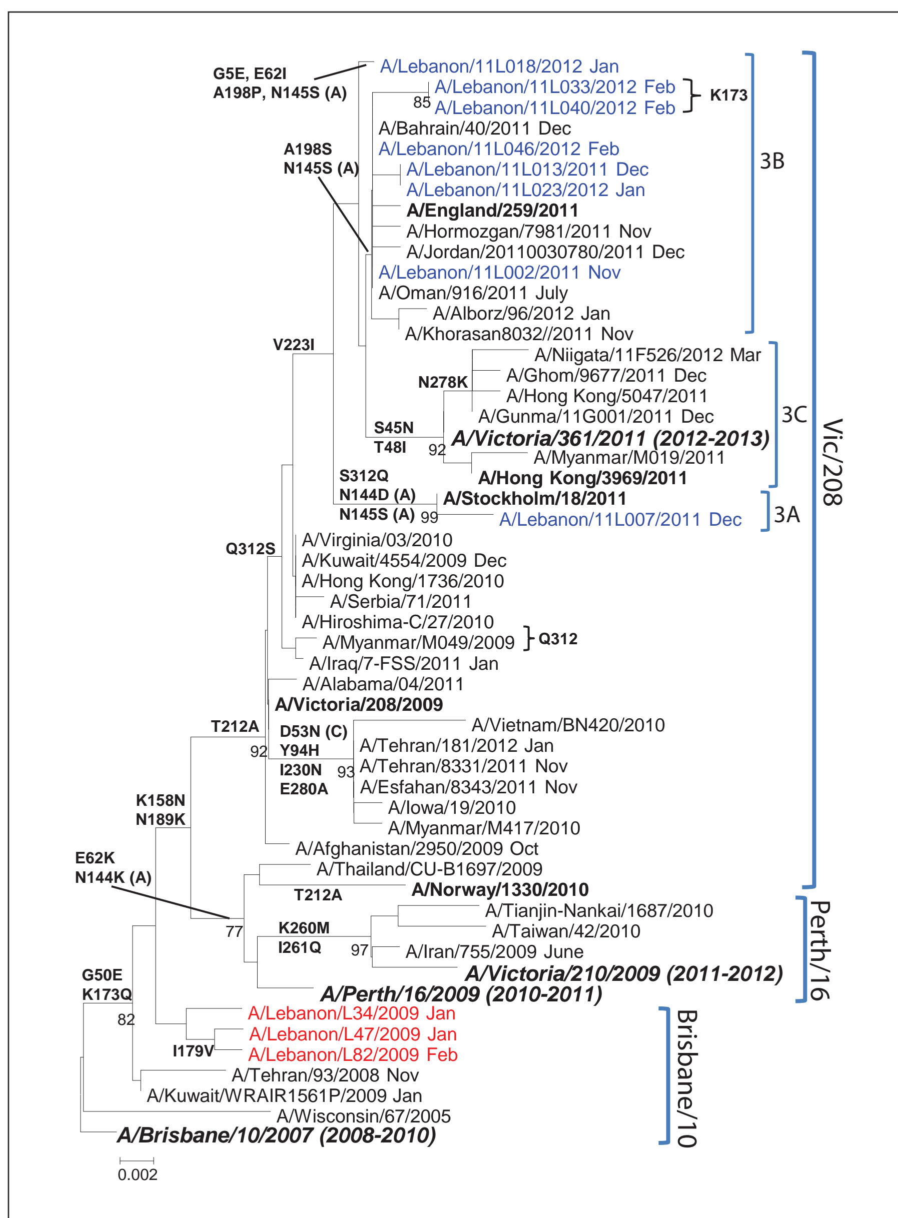


**Fig. 4.** Phylogenetic analysis of the hemagglutinin (HA1) genes of influenza A/H3N2 viruses isolated in Lebanon. The trees were constructed based on the nucleotide sequences using the Neighbor-Joining method with bootstrap analysis of 1,000 replicates. A/Brisbane/10/2007 was used as the root for the tree and bootstrap values greater than 70% are shown. Amino acid mutations characteristic of the main clusters or the Lebanese isolates are indicated on the branches. The HA antigenic site to which a mutation belongs is in parentheses. Vaccine strains are in bold italics and reference strains are in bold. Lebanese isolates from 2008–2009 and 2011–2012 seasons are shown in red and blue, respectively.

work during the pandemic [11]. In the case of H3N2, all viruses from the 2011–2012 season sequenced in this study belonged to the A/Victoria/208/2011-like lineage, and most of them fell into clade 3B. The Lebanese isolates closely resembled WHO-designated reference strains A/Stockholm/18/2011 and A/England/259/2011 which exhibited 64- and 32-fold reduction, respectively, in their HA inhibition (HAI) titers of sera raised against the A/Victoria/210/2009 vaccine strain [17]. These viruses accumulated several mutations, including 3–4 in the antigenic sites compared with the vaccine strain which explains the antigenic drift marked reduction in their HAI titers.

The prevalence of oseltamivir-resistant H1N1p viruses remained low worldwide in the post-pandemic period, albeit at a slight increase from their level during the pandemic period [24–26]. Nonetheless, the rate of oseltamivir resistance is much higher among high-risk groups such as immunocompromised patients, and clusters of high community transmission rates have been reported [25, 27]. Hurt et al. [28] reported a significant increase in the frequency H275Y oseltamivir-resistant H1N1p virus-

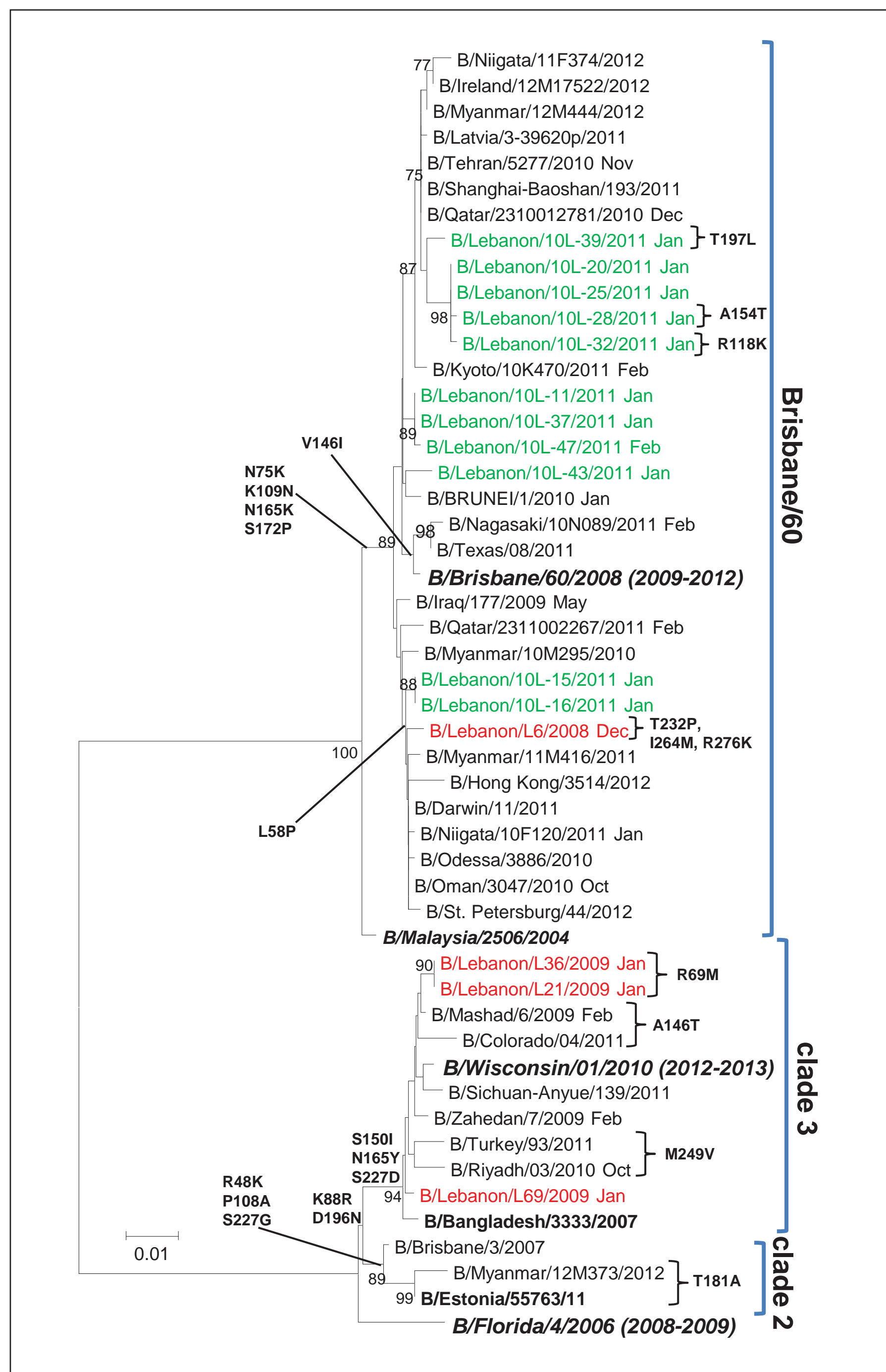


**Fig. 5.** Phylogenetic analysis of the hemagglutinin (HA1) genes of influenza B viruses isolated in Lebanon. The trees were constructed based on the nucleotide sequences using the Neighbor-Joining method with bootstrap analysis of 1,000 replicates. The trees are rooted in the middle and bootstrap values greater than 70% are shown. Amino acid mutations characteristic of the main clusters or the Lebanese isolates are indicated. Vaccine strains are in bold italics and reference strains are in bold. Lebanese isolates from 2008–2009 and 2010–2011 seasons are shown in red and green, respectively.

es in a community in Australia. These resistant viruses had three additional NA mutations, V241I, N369K, and N386S, that were suggested to compensate for the destabilizing effect of the H275Y mutation and could, therefore, be permissive for fixing the H275Y mutation in the population. All of the 2011–2012 season H1N1p viruses isolated in Lebanon, despite lacking the H275Y mutation, had these potentially permissive mutations, and 55% of the 2010–2011 isolates had 2 of these mutations (V241I and N369K). Collectively, these data suggest that H1N1p viruses have the capacity to accommodate the H275Y with potentially minimal burden on their fitness.

We also detected one H1N1p isolate with a 4-fold reduced oseltamivir $IC_{50}$, due to an NA-S247N mutation, in a patient prior to prescription of oseltamivir. This mutation was reported in 10 and 30% of H1N1p viruses isolated in Australia and Singapore, respectively, during the same period [20]. The Lebanese S247N isolate was phylogenetically distinct from those isolated in Australia and Singapore. Of note, this mutation was also reported in H5N1 viruses isolated in Laos, reducing their susceptibility to oseltamivir by 24-fold [29]. These reports suggest that the S247N mutation could be naturally selected without antiviral drug pressure and is transmissible. While the $IC_{50}$ values of S247N viruses are well below the biological drug concentrations of oseltamivir [20], the possibility that this mutation gets selected in viruses with the H275Y mutation is of concern. An isolate with dual NA-S247N and -H275Y mutations has been already reported and was shown to result in a 6,000-fold increase in oseltamivir $IC_{50}$ [20], rendering treatment with oseltamivir ineffective.

In conclusion, given the diversity of influenza viruses and the potential for rapid emergence of antiviral drug-resistant strains, it is essential to closely monitor influenza viruses both by phenotypic and genotypic assays to quickly identify such emerging mutations. Since the worldwide distribution of antiviral drug-resistant influenza strains varies, it is important to strengthen influenza surveillance at local and regional levels. Such information is critical for maintaining the effectiveness of antiviral drugs for both prophylaxis and treatment of influenza infections.


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## Disclosure Statement

The authors have no conflicts of interest to disclose.

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