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Research Paper

Molecular epidemiology and genetic characterization of influenza B virus in Lebanon during 2016–2018

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ABSTRACT

Background: Influenza B viruses are a major cause of serious acute respiratory infections in humans.
Methods: Nasopharyngeal swabs were collected from subjects with influenza-like illness during October 2016–June 2018 and screened for influenza A and B. The hemagglutinin (HA) and neuraminidase (NA) genes of the Lebanese influenza B specimens were sequenced and phylogenetically compared with the vaccine strains and specimens from the Eastern Mediterranean Region and Europe.
Results: Influenza A and B viruses co-circulated between October and May and peaked between January and March. During the 2016–2017 season, A/H3N2 (33.4%) and B/Yamagata (29.7%) were the predominantly circulating viruses followed by B/Victoria and A/H1N1pdm09 viruses. During the 2017–2018 season, A/H3N2 (31.5%) and A/H1Npdm09 (29.3%) were most prevalent with co-circulation of B/Yamagata and to a lesser extent B/Victoria viruses. The B/Yamagata specimens belonged to clade-3 while the B/Victoria belonged to clade-1A. None of the analyzed specimens had a mutation known to confer resistance to NA inhibitors (NAIs).
Conclusion: Multiple subtypes of influenza co-circulate each year in Lebanon with a peak between January and March. The trivalent vaccine included a B/Victoria strain which mismatched the B/Yamagata lineage that predominated during the study period, highlighting the importance of quadrivalent vaccines.

1. Introduction

Influenza virus is a significant public health threat that contributes to high levels of morbidity and mortality globally. It was recently estimated that influenza causes between 290,000–650,000 deaths annually (Troeger et al., 2019). Influenza virus belongs to the family of *Orthomyxoviridae* which contains four types A, B, C and D classified according to variations in matrix protein and nucleoprotein antigens (Moghadami, 2017; Pleschka, 2013; Bedford et al., 2015). Two influenza A subtypes, A/H1N1 and A/H3N2, and two influenza B lineages, B/Yamagata and B/Victoria, circulate among humans globally. Influenza A virus has a broad host range (humans, birds, horses, swine, etc.) and has the potential to cause pandemics by antigenic shift. Unlike influenza A virus, influenza B possesses limited diversity and infects a limited host range (i.e., humans and seals) (Chen et al., 2007; Cox and Subbarao, 2000; Bodewes et al., 2013).

Influenza B virus (influenza B/Lee/40) was first isolated in 1940. Since 1983, influenza B was classified into two distinct lineages as B/Victoria and B/Yamagata lineages based on the HA antigen (Kanegae et al., 1990; Ali et al., 2014). The two lineages have been reported to genetically evolve by reassortment events between gene segments or by antigenic drift due to selection pressure to escape prevailing host

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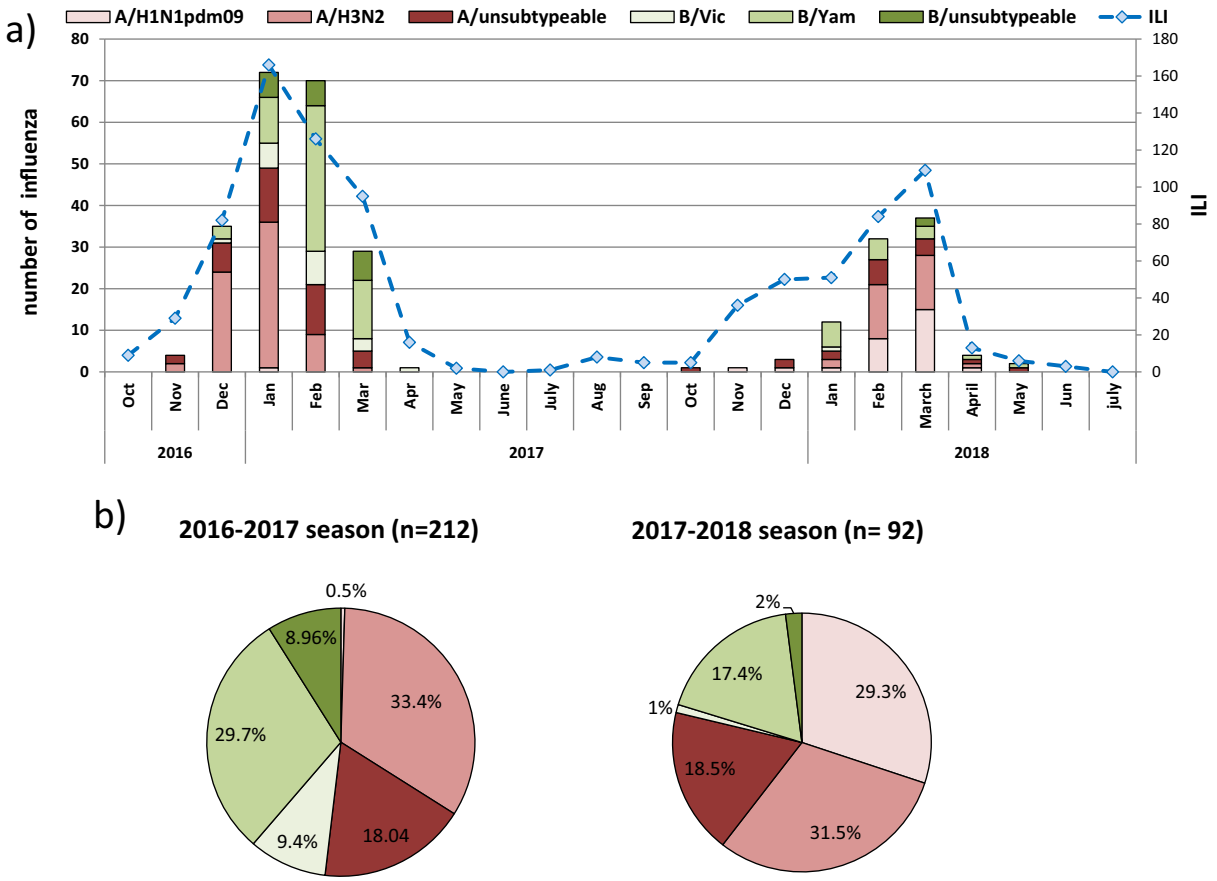

Fig. 1. Distribution of influenza viruses in Lebanon. (a) Monthly distribution of ILI and influenza-confirmed cases (b) Incidence of influenza by subtype and lineage during the 2016–2017 and 2017–2018 seasons.

immunity (McCullers et al., 2004). Both lineages have co-circulated in many regions of the world with different regional dominance. This has posed a challenge for the prediction of the strain to be included in the trivalent influenza vaccine (Langat et al., 2017). Recently, a recommendation was made to switch to a quadrivalent vaccine that includes two influenza A and the two influenza B viruses, representing both the Yamagata and Victoria lineages (Ambrose and Levin, 2012).

Neuraminidase inhibitors (NAIs) such as oseltamivir and zanamivir are the drugs of choice in treating influenza B infections. However, mutations, particularly those occurring in the catalytic or framework residues of the neuraminidase (NA) protein, can result in resistance to this class of antivirals (Okomo-Adhiambo et al., 2013).

In this study, we investigated the incidence of influenza B viruses in Lebanon during two recent influenza seasons 2016–2017 and 2017–2018. Furthermore, we performed genetic characterization of the hemagglutinin (HA) and neuraminidase (NA) genes and screened the latter for genetic markers that confer resistance to NAIs.

2. Materials and methods

2.1. Ethical statement

The study was approved by the Institutional Review Board of the American University of Beirut (project approval number: PED.GD.02) and written informed consent was signed by participants or their guardians at the time of sample collection.

2.2. Study population and sample collection

The surveillance was conducted from October 2016 till June 2018 covering 2016–2017 and 2017–2018 influenza seasons at five sites distributed across Lebanon. Nasopharyngeal swabs were collected from subjects of all ages diagnosed with influenza-like symptoms: fever ($\geq 38\,^{\circ}C$) and cough onset within the last 10 days. Swabs were suspended into viral transport media and stored at $-20\,^{\circ}C$. Information about the patient's age and their vaccination status was collected.

2.3. RNA extraction and influenza virus detection

Viral RNA was extracted using PureLink® RNA Mini Kit (Invitrogen) following the manufacturer's instructions. Influenza A and B viruses were detected by real-time multiplex PCR using AgPath-ID™ One-Step RT-PCR (Applied Biosystems) according to the protocols established by the World Health Organization (WHO) (World Health Organization, 2014). Influenza positive specimens were further subtyped into A/H1N1pdm09, A/H3N2, B/Victoria, and B/Yamagata by using real-time PCR.

2.4. Gene sequencing and phylogenetic analysis

The HA and NA genes of influenza B positive specimens with Cq values < 30 were amplified by QIAGEN One-Step RT-PCR using previously published primers (Dapat et al., 2009; Tewawong et al., 2015). The resultant PCR products were sequenced by Sanger sequencing at Macrogen Inc. Multiple sequence alignments were performed using Clustal W implemented in the BioEdit program 7.0 (Hall, 1999). Phylogenetic trees were constructed using Mega 7.0 software by applying the Maximum Likelihood method with the best fit model and 1000 bootstrap replicates (Kumar et al., 2016). The analysis included representative sequences from different regions of the world available in

Global Initiative on Sharing All Influenza Data (GISAID) and the WHO recommended influenza virus vaccines for the 2016–2017 and 2017–2018 northern hemisphere influenza season. Nucleotide sequences were submitted to NCBI with accession numbers MK817658-MK817674 for influenza B/Yamagata HA genes, MK817681-MK817682 for influenza B/Victoria HA gene, MK828540-MK828555 for influenza B/Yamagata NA genes, and (MK817679) for influenza B/Victoria NA genes.

2.5. Glycosylation analysis

Potential N-glycosylation sites were predicted in the HA and NA glycoproteins using the NetNGlyc Server 1.0, where a threshold ≥0.5 represents a predicted glycosylation site (Gupta et al., 2004).

3. Results

3.1. Influenza seasonality and subtype incidence

A total of 889 of nasopharyngeal swabs were collected between October 2016 and July 2018 from subjects with ILI and an age range of 0.5–65 years (median = 2 years). Among these, 28% and 30.7% reported receiving the influenza vaccine within 2016–2017 and 2017–2018 influenza seasons, respectively. Overall, 34% (n = 304) of the cases were influenza-positive. All influenza cases occurred between October and May, with peak activity between January and March (Fig. 1a). During the 2016–2017 influenza season, 519 samples were analyzed, and out of these, 40.8% (n = 212) were positive for influenza virus; 52% (n = 110) were influenza A, and 48% (n = 102) were influenza B. Further characterization of the detected influenza specimens revealed that A/H3N2 (n = 71; 33.4%) and B/Yamagata (n = 63; 29.7%) viruses predominated (Fig. 1b). During the 2017–2018 season, out of 370 collected samples, 24.8% (n = 92) were influenza-positive among which 79.3% (n = 73) were influenza A and 20.6% (n = 19) were influenza B. Analysis of the influenza-positive specimens revealed dominance of A/H3N2 (n = 29; 31.5%) and A/H1N1pdm09 (n = 27; 29.3%) viruses (Fig. 1b).

3.2. Phylogenetic characterization of the Lebanese influenza B strains

3.2.1. Genetic analysis of the HA gene segments

The nucleotide sequences of the HA gene were obtained for 19 influenza B samples. Additional 16 influenza B HA sequences that were reported from Lebanon between 2016 and 2018 were retrieved from GISAID and included in the phylogenetic analysis. Among the 35 HA sequences, 29 (82.8%) were of the Yamagata lineage, and 6 (17.2%) belonged to the Victoria lineage.

Three antigenically distinct clades have been designated for the influenza B: clade 1, the B/Brisbane/60/2008-like clade; clade 2, the B/Massachusetts/2/2012-like clade; clade 3, the B/Wisconsin/1/2010-like clade. Phylogenic analysis of the 29 HA gene sequences from Lebanon revealed that the influenza B/Yamagata viruses belonged to clade 3 and formed two monophyletic groups, Yamagata 3a and Yamagata 3b (Fig. 2a). These viruses shared an amino acid similarity of 90% with influenza B/Phuket/3073/2013, the 2016–2017 and 2017–2018 northern hemisphere vaccine strain.

We next examined the substitutions among the Lebanese specimens including those occurring in the antigenic epitopes: 120-loop (residues 116–137), 150-loop (141–150), 160-loop (162–167), and 190-helix (194–202) (Fig. 3). Compared with B/Phuket/3073/2013, viruses belonging the clade 3 shared three amino acid substitutions (L172Q, M251V, and D196N, a potential N-glycosylation site in the 190-helix). Twenty of the Lebanese strains from both seasons belonged to subclade 3a as the reference strain B/Mauritius/1791/2017. This subclade also accommodated the majority of reported B/Yamagata viruses from the Eastern Mediterranean Region (EMR). Two of the 2017–2018 Lebanese specimens within this cluster were characterized by an additional substitution D232N that was also detected among viruses from neighboring countries (Kuwait and the United Arab Emirates) from the same season. One specimen was distinguished from the other viruses by a D163N substitution in its 120-loop. Subclade 3b included nine strains from the 2016–2017 and 2017–2018 influenza seasons and did not possess any unique amino acid substitutions.

The six influenza B/Victoria viruses belonged to the clade 1A, represented by reference strain B/Ireland/3154/2016. This clade also harbored strains from neighboring EMR countries and Europe (Norway and Ukraine) (Fig. 4a). The Lebanese viruses possessed > 98% amino acid similarity with B/Brisbane/60/2008, the influenza B/Victoria vaccine strain recommended for 2016–2017 and 2017–2018 influenza seasons. Comparison of all clade 1A viruses with the vaccine strain revealed an N129D substitution in the 120-loop. These strains also possess an additional amino acid substitution (D197N), which introduces a potential glycosylation site in the 190-helix epitope. Two Lebanese viruses isolated during the 2016–2017 season could be differentiated by an additional substitution each (N145D and G334E). An additional I117V substitution (not shown on the tree due to shorter sequences for some viruses) was identified in the 120-loop among most of the B/Victoria viruses isolated during the 2016–2017 and 2017–2018 seasons.

3.2.2. Genetic analysis of the NA gene segments

The NA genes were successfully sequenced from 17 influenza B specimens identified in this study. In addition, NA sequences for another 16 Lebanese viruses from the same period were downloaded from GISAID. Phylogenetic analysis of the NA genes from the 33 Lebanese influenza B strains classified them into two distinct clades 1 and 3, corresponding to their HA phylogenetic tree topology. Twenty-eight (85%) of the influenza B/Yamagata specimens belonged to clade 3 whereas five (15%) of influenza B/Victoria lineage NA genes belonged to clade 1A. No inter- or intra-clade reassortment events were detected among the Lebanese influenza B strains.

Similar to the HA phylogeny, the NA genes of clade 3 B/Yamagata viruses fell into two subclades, 3a and 3b, represented by (B/Mauritius/1791/2017) and (B/Phuket/3073/2013) reference strains, respectively. Eighteen of the sequenced Lebanese B/Yamagata viruses identified during the 2017–2018 season clustered in subclade 3a. The viruses within this cluster were characterized by a K373Q substitution compared to the vaccine strain (Fig. 2b). Sixteen out of the 18 B/Yamagata viruses were genetically related to the reference strain B/Mauritius/1791/2017 and were characterized by four additional substitutions (I49M, R65H, I171M, and D342K) along with viruses from neighboring EMR countries (Oman, Kuwait, United Arab Emirates, Saudi Arabia, Jordan, and Qatar) isolated during 2017 and 2018. Two viruses belonging to this cluster were each characterized by a unique amino acid substitution (G104R and E338G). Another two viruses within this subclade were from the 2016–2017 influenza season were characterized by two unique amino acid substitutions (G70R and D342N). The remainder 10 B/Yamagata Lebanese viruses from the 2016–2017 belonged to subclade 3b that is characterized by amino acid substitutions (S295R and I49T) compared to the vaccine strain. The S295R substitution was similar to clade 2 reference strain (B/Estonia/55669/2011). One Lebanese subclade 3b virus carried a T106 N, that was previously identified in the southern hemisphere during 2011 to cause a mild reduction in oseltamivir susceptibility (Okomo-Adhiambo et al., 2013). Subclade 3b also harbored influenza B viruses from Europe (Norway, Netherlands, Turkey, and England) and the EMR (Tunisia, Oman, and Saudi Arabia) collected during 2016 and 2017.

Comparison with (B/Brisbane/60/2008) vaccine strain revealed that all the Lebanese influenza B/Victoria viruses (n = 5) belonged to clade 1A and shared the same branching point with influenza B viruses reported from Oman, Egypt, and the United Arab Emirates. All five specimens harbored (I120V, K220 N, S295R, N340D, D384G, and

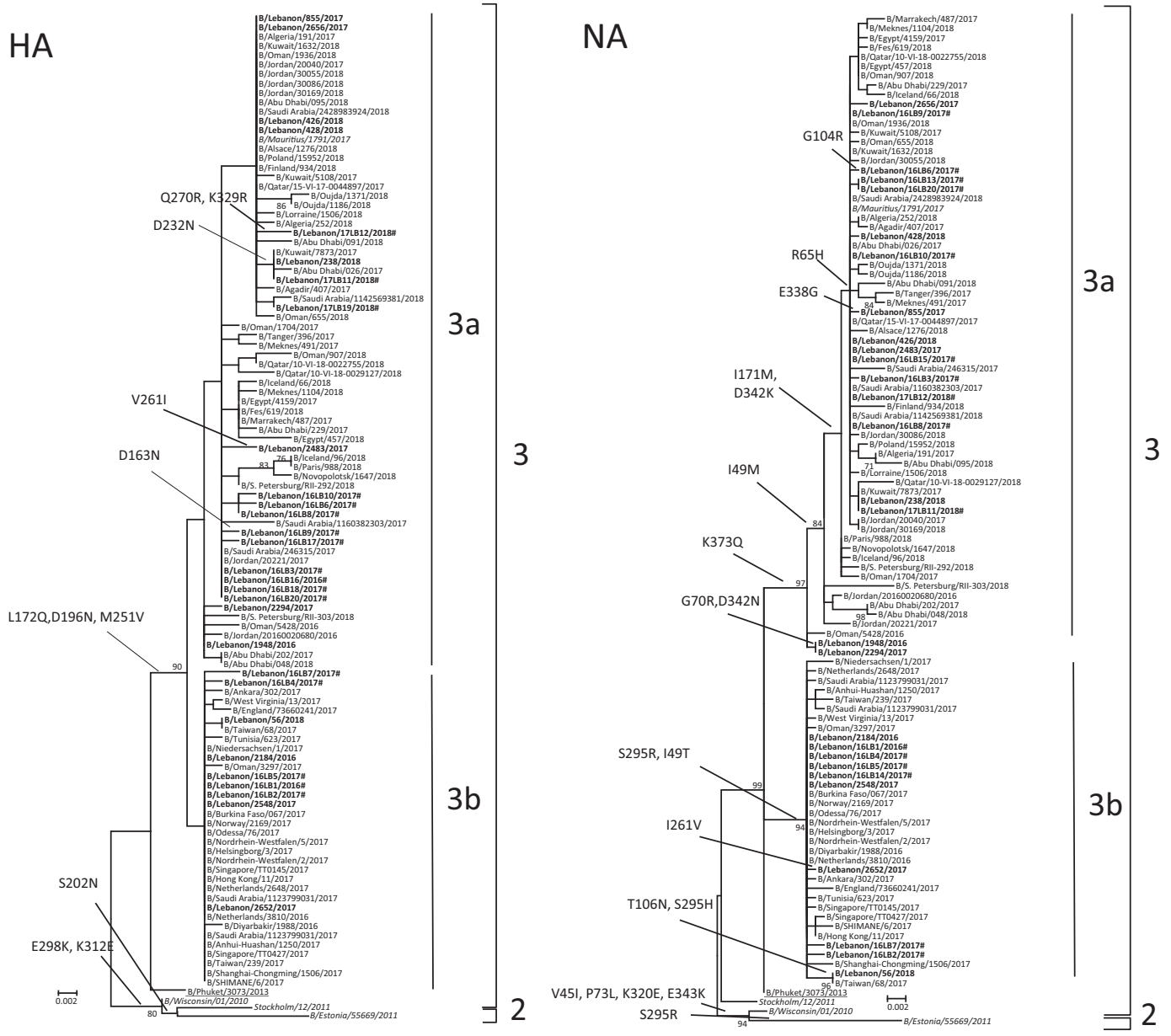

Fig. 2. Phylogenetic analysis of the HA and NA nucleotide sequences of influenza B/Yamagata lineage viruses detected in Lebanon between 2016 and 2018. The phylogenic trees were constructed using the Maximum Likelihood method analysis with 1000 bootstrapped replicates based on the best-fit nucleotide substitution model. The Hasegawa-Kishino-Yano model was used for HA and Tamura 3-parameter model for NA tree. The bootstrap support values (> 70%) for each node are shown. The sequences of the WHO recommended vaccine strain for the 2016–2017 and 2017–2018 influenza seasons along with other reference strains and viruses reported from Lebanon and neighboring countries were obtained from the Global Initiative on Sharing all Influenza Data (GISAID) and included in the analysis for comparison. The vaccine strain is underlined, B/Phuket/3073/2013. The Lebanese strains are shown in bold; #, indicates viruses sequenced in this study. The reference strains for clade designation are italicized.

E358K) substitutions in the NA protein. Two of these viruses had an additional (V401I) substitution that was also reported in the EMR and Europe (Fig. 4b). None of the substitutions detected in the NA segment were previously associated with resistance to NAIs.

4. Discussion

In this study, the incidence of influenza among subjects showing ILI in Lebanon was 34% between 2016 and 2018. Molecular characterization of the viruses circulating in Lebanon showed that influenza A/H3N2 and B/Yamagata were the predominant circulating viruses among ILI patients during the 2016–2017 season. Similarly, A/H3N2 was the predominant subtype among patients with severe acute respiratory infections (SARI) according to the data reported by the National Influenza Center in Lebanon (Ministry of Public Health Lebanon). Influenza A/H1N1pdm09 circulated at a very low level (< 1%) in Lebanon, the EMR, and Europe until the beginning of the 2017–2018 season (European Centre for Disease Prevention and Control, 2018). However, it was the predominantly circulating subtype in Iraq (61%) during the same period (World Health Organization, 2017, 2019). During the following 2017–2018 season, influenza A/H1N1pdm09 and A/H3N2 viruses prevailed in Lebanon reflecting what was also observed in some European and Asian countries (Belarus, Kazakhstan and the Russian Federation). In contrast, the A/H1N1pdm09 was the most prevalent subtype (59%) in most Arab countries in Northern Africa (Algeria, Egypt, and Tunisia) and Western

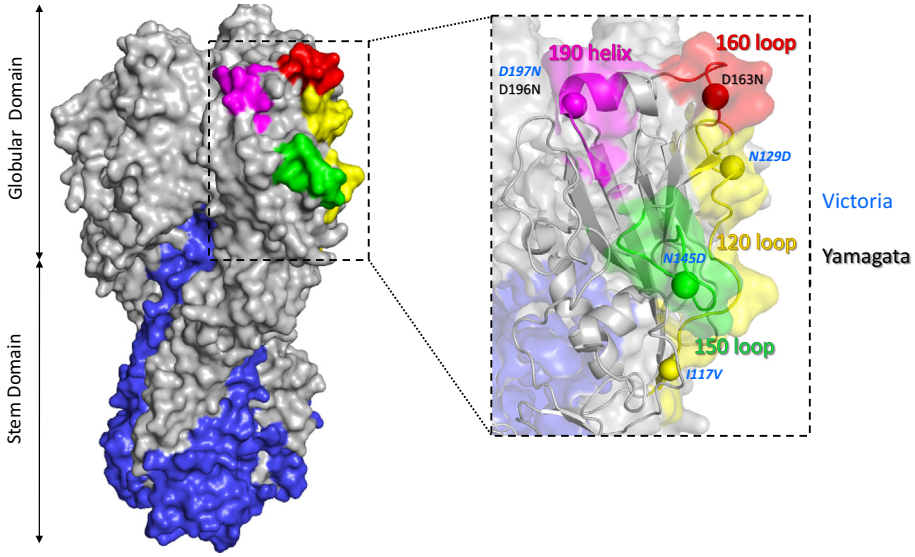

Fig. 3. Structural representation of the hemagglutinin protein (PDB ID: 4FQM). The HA1 subunit is shown in grey and the HA2 subunit is shown in blue. The amino acid differences in the antigenic epitopes are shown in black fonts for B/Yamagata and blue for B/Victoria. The structure was generated in PyMol software. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Asia (Iraq, Jordan, Kuwait, Oman, and Qatar) (World Health Organization, 2018a).

During the study seasons, the trivalent influenza vaccine, which includes a B/Victoria vaccine strain (B/Brisbane/60/2008-like virus) was used in Lebanon. However, B/Yamagata was the dominant strain in Lebanon and other EMR countries (World Health Organization, 2017). This highlights the importance of introducing the quadrivalent vaccine that includes both lineages of influenza B and thus offering better protection. The phylogenetic analysis revealed that the influenza B strains circulating in Lebanon belonged to the Yamagata clade 3 and Victoria clade 1A, with a predominance of the former clade.

The HA1 domain of influenza B includes four major antigenic epitopes: 120-loop (116–137), 150-loop (141–150), 160-loop (162–167), and 190-helix (194–202) (Shen et al., 2009). Amino acid changes within these regions alter the virus antigenicity. All circulating Yamagata 3 and Victoria 1A strains shared an amino acid substitution (D196/7N) in the 190-helix compared to the respective vaccine strains, B/Phuket/3073/2013 and B/Brisbane/60/2008, respectively. This substitution introduces a potential N-linked glycosylation site in the 190-helix that could significantly interfere with binding by neutralizing antibodies (Kishida et al., 2012) (Chen et al., 2008). The same substitution was previously detected among B/Yamagata and B/Victoria viruses reported from Saudi Arabia between 2010 and 2011 and East Asia between 2012 and 2015 (Ali et al., 2014; Hibino et al., 2018). The introduction of an N-glycosylation at this residue was shown to alter both the receptor binding specificity and antigenicity of influenza B viruses (Saito et al., 2004). The Victoria clade 1A strains possess two additional mutations (I117V and N129D) in the 120-loop, previously reported in China during the 2015–2017 seasons (Lei et al., 2019). Serological characterization against viruses with similar substitutions by hemagglutination inhibition assay did not reveal any major antigenic differences with respect to the recommended vaccine strain (European Centre for Disease Prevention and Control, 2017; Tsedenbal et al., 2018). However, recently characterized B/Victoria strains from the 2018–2019 also possess the I117V and N129D substitutions accompanied by the deletion of three (Δ162–164) or two (Δ162–163) amino acid residues in 160-loop. These strains were poorly recognized by antisera raised against B/Brisbane/60/2008 but were relatively close to B/Colorado/06/2017. Thus, it was recommended to include the latter in the trivalent vaccine strain for 2018–2019 influenza season while maintaining the Yamagata vaccine strain from the previous seasons for the quadrivalent vaccine (European Centre for Disease Prevention and Control, 2018).

NAIs are the most commonly used drugs for the prevention and treatment of influenza virus infections (Matrosovich et al., 2004). The NA active site contains conserved amino acids residues (R116, D149, R150, R223, E275, R292, R374, and Y409) that are in direct contact with sialic acid receptors. This enzyme active domain is supported by framework residues (E117, R154, W177, S178, D197, I221, E226, H273, E276, N293, and E428) that provide a backbone for the catalytic site. Amino acid substitutions at these sites can confer resistance to NAIs (Burnham et al., 2014). Global data reported on influenza B susceptibility during the 2016–2017 season estimated that 0.4% of B/Victoria viruses and 0.2% of B/Yamagata viruses exhibited reduced susceptibility to at least one NAI (Lackenby et al., 2018). However, none of the influenza B viruses analyzed during the 2017–2018 season displayed resistance or reduced susceptibility to any of the NAIs (Zhu et al., 2019; Chow et al., 2018). In our study, none of the Lebanese viruses possessed any of the changes in the NA that were reported to confer resistance to NAIs (World Health Organization, 2018b). One specimen, B/Lebanon/56/2018, had a T106 N substitution in the NA that was previously reported in a strain with reduced susceptibility to oseltamivir (Okomo-Adhiambo et al., 2013).

5. Conclusion

This study revealed co-circulation of multiple influenza subtypes in Lebanon during two consecutive influenza seasons with a mismatch between circulating strains and the recommended vaccine strain. Continuous surveillance using molecular assays enable us to better understand influenza virus epidemiology and evolutionary dynamics and to maintain the effectiveness of current antivirals. Furthermore, effective interventions and advocacy are crucial to improving influenza vaccine uptake and acceptance to reduce the disease burden.

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Declaration of Competing Interest

None declared.

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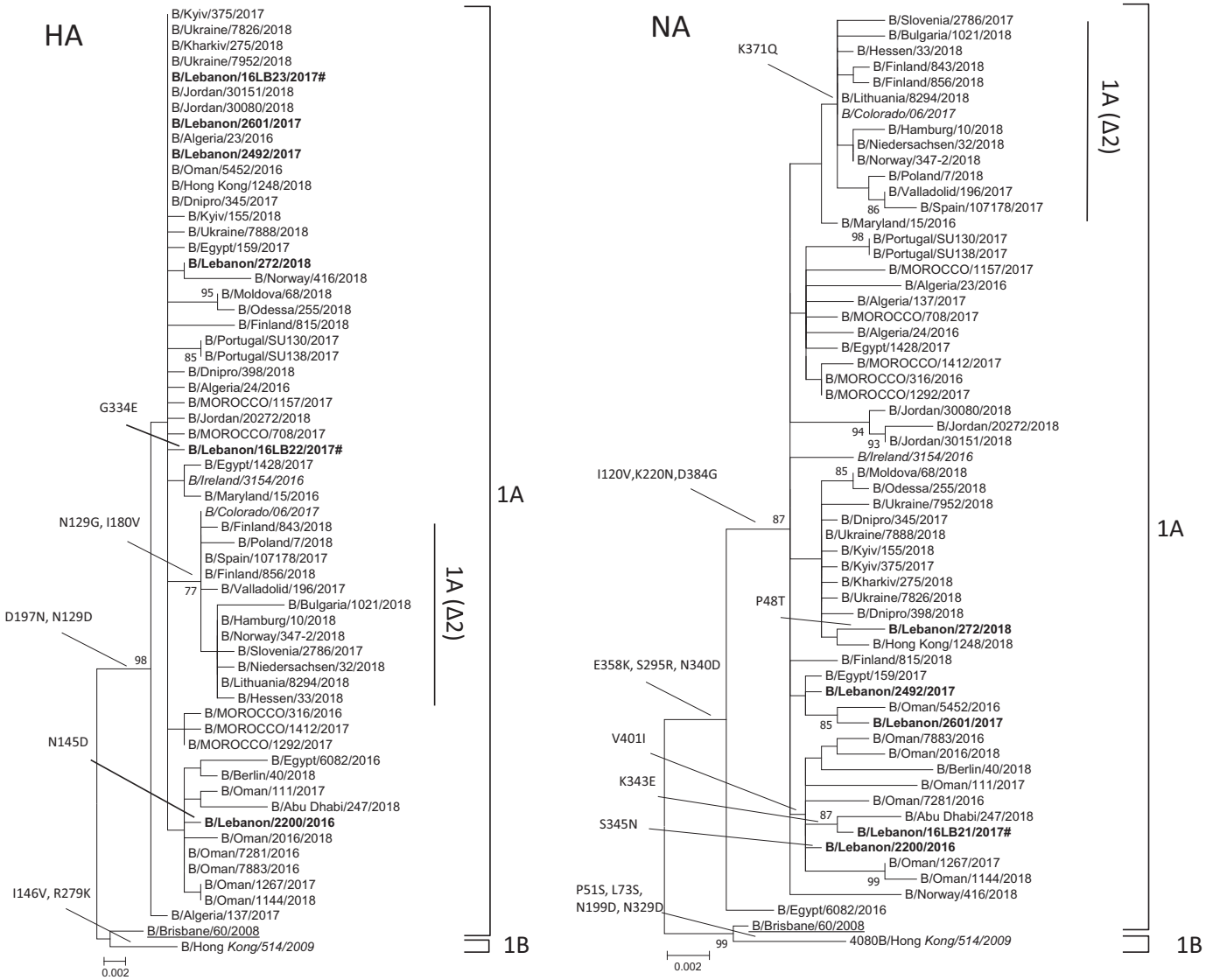

Fig. 4. Phylogenetic analysis of the HA and NA nucleotide sequences of influenza B/Victoia lineage viruses detected in Lebanon between 2016 and 2018. The phylogenic trees were constructed using the Maximum Likelihood method analysis with 1000 bootstrapped replicates based on the best-fit nucleotide substitution model. The Hasegawa-Kishino-Yano model was used for both the HA and NA trees. The bootstrap support values (> 70%) for each node are shown. The sequences of the WHO recommended vaccine strain for the 2016–2017 and 2017–2018 influenza seasons along with other reference strains and viruses reported from Lebanon and neighboring countries were obtained from the Global Initiative on Sharing all Influenza Data (GISAID) and included in the analysis for comparison. The vaccine strain is underlined, B/Brisbane/60/2008. The Lebanese strains are shown in bold; #, indicates viruses sequenced in this study. The reference strains for clade designation are italicized.

participating in the study.

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