Research Article submitted to *Virus Evolution*:

Differential Disease Severity and Whole Genome Sequence Analysis for Human Influenza A/H1N1pdm Virus in 2015-2016 Influenza Season

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Abstract: 232/350

Main Text: 3571/10000

Abstract:

During the 2015-16 winter, the US experienced a relatively mild influenza season compared to Taiwan which had a higher number of total and severe cases. While H1N1pdm viruses dominated global surveillance efforts that season, the global distribution of genetic variants and their contributions to disease severity have not been investigated. Samples collected from influenza A positive patients by the Johns Hopkins Center of Excellence for Influenza Research and Surveillance (JH-CEIRS) active surveillance in the emergency rooms in Baltimore, Maryland, USA and northern Taiwan between November 2015 and April 2016, were processed for influenza A virus whole genome sequencing. In Baltimore, the majority of the viruses were the H1N1pdm clade 6B.1 and no H1N1pdm clade 6B.2 viruses were detected. In northern Taiwan, more than half of the H1N1pdm viruses were clade 6B.1 and 38% were clade 6B.2, consistent with the global observation that most 6B.2 viruses circulated in Asia and not North America. Whole virus genome sequence analysis identified two genetic subgroups present in each of the 6B.1 and 6B.2 clades and one 6B.1 intraclade reassortant virus. Clinical data showed 6B.2 patients had more disease symptoms including higher crude and inverse probability weighted odds of pneumonia than 6B.1 patients, suggesting 6B.2 circulation may contribute to the severe flu season in Taiwan. Local surveillance efforts linking H1N1pdm virus sequences to patient clinical and demographic data improve our understanding of influenza circulation and disease potential.

63 Key words: influenza, H1N1pdm, whole genome sequencing, disease severity, Taiwan,

64 Baltimore

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Introduction:

The 2009 pandemic H1N1 Influenza A virus (H1N1pdm) has supplanted the previous human H1N1 viruses to become the seasonal human H1N1 virus. In the 2015-16 influenza season, the northern hemisphere experienced its second global H1N1pdm-dominant year since 2009. The 2015-16 Influenza activity in the US was considered moderate, with lower numbers of total cases and a later peak epidemic when compared with the previous three influenza seasons [1]. However, in Taiwan, the 2015-16 season was much more serious [2], with an earlier start and higher numbers of total as well as severe, influenza cases compared to the prior influenza season 2014-15 [3].

Influenza A viruses (IAV) are subdivided into subtypes based on the antibody response to the viral glycoproteins, hemagglutinin (HA) and neuraminidase (NA). Viruses are further divided into genetic clades based on HA sequences in order to monitor for mutations that might lead to antigenic drift. The 2015-16 northern hemisphere vaccine strain of H1N1pdm, A/California/7/2009, is defined as H1 clade 1. Prior to the 2015-16 season, H1N1pdm was dominated by clade 6B. The global rise of H1 clade 6B.1 carrying amino acid changes S84N, S162N and I216T from clade 6B was detected in human surveillance efforts starting in June 2015 and is now the dominant H1N1 clade. The H1 clade 6B.2 viruses (V152T, V173I of HA1 and E164G and D174E of HA2) emerged in July 2015, peaked in January 2016, were found primarily in Asia and disappeared from surveillance efforts at the end of 2016 [4, 5]. The majority of 6B.1 and 6B.2 viruses were antigenically similar to the recommended components of the 2015-16 Northern Hemisphere H1N1pdm influenza vaccine A/California/7/2009 [1, 3, 6, 7],

although antigenic difference between egg-adapted and circulating virus strains may have contributed to increased numbers of cases [8].

The genome of IAV consist of 8 segments of negative-sense RNA. In addition to HA mutations, mutations in the other 7 viral gene segments also result in genetic variants which can alter virus fitness, subvert pre-existing immunity and/or reduce vaccine effectiveness. Any of these changes could potentially alter disease severity or total number of cases [9]. Next-generation sequencing provides a sensitive and rapid method for sequencing of viruses directly from human specimens without any need for virus isolation or culture – both of which are known to select for viruses bearing additional mutations [10].

Most current global influenza surveillance systems lack connections between viral sequences and detailed patient demographic and clinical data. The Johns Hopkins Center of Excellence for Influenza Research and Surveillance (JH-CEIRS) has been actively performing human influenza surveillance since 2014. Surveillance efforts involve the connection of viral genome data and isolates with patient demographic and clinical data to gain more insights into how virus genetic variation can affect clinical disease. We hypothesized that sequence differences in influenza viruses could be associated with distinct clinical outcomes. This study involved concurrent, active influenza surveillance during the 2015-16 season in Baltimore, USA and northern Taiwan to compare and contrast differential epidemics in the same year between the

two geographic regions and to analyze possible disease severity with influenza viral genotypes.

Materials and Methods:

Active surveillance enrollment and sample collection

Institutional Review Boards at the Johns Hopkins University School of Medicine and Chang Gung Memorial Hospital provided ethical approval of the study (IRB00052743). From November 2015 to April 2016, active surveillance was performed in the adult emergency departments of the Johns Hopkins Medical Institutions (JHMI) at the East Baltimore and Bayview campuses in Baltimore, Maryland, USA and Chang Gung Memorial Hospitals (CGMH) in the northern Taiwan metropolitan area, including hospitals in the Taipei, Linkou, and Keelung Branches. Influenza-like illness was defined as documented or reported $\geq 38^{\circ}\text{C}$ fever and any of one of three respiratory symptoms including cough, headache and/or sore throat within the past 7 days. Exclusion criteria included subjects who are unable to speak and understand English (in US) or Mandarin (in Taiwan), unable to provide written informed consent, currently incarcerated, or previously enrolled in the study during the same influenza season. Patients were approached by trained clinical coordinators who obtained written, informed consent before collected specimens and demographic and clinical data using a standard questionnaire. Data was confirmed by examination of the patient's electronic health record. All data was de-identified and stored in a secure REDCap database [11, 12].

Diagnosis and subtyping

Nasopharyngeal swabs or nasal washes of patients collected at the time of enrollment were tested for influenza A virus infection using the Cepheid Xpert Flu/RSV test (Cepheid, Sunnyvale, CA). Samples that were influenza A virus positive were further subtyped by qRT-PCR with specific H1 or H3 primers and probes according to USA Centers for Disease Control and Prevention (CDC) protocols. Only H1N1pdm positive samples were moved on to whole genome sequencing (WGS) analysis.

Whole genome sequencing

Viral RNA was isolated from the influenza A positive clinical samples using the MagMax Viral RNA isolation reagent (Thermo Fisher) on an Eppendorf epMotion 5073 liquid handling workstation. Samples were processed for WGS using multi-segment PCR [10] and then prepared for deep sequencing using the Nextera XT library preparation reagent (Illumina). Samples were sequenced on the Illumina NextSeq platform. Raw paired-end data were first processed through Trim galore! (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) with a quality score of 30 and an adapter sequence of CTGTCTCTTATACACATCT, only retaining pairs that both passed through this quality control step. The quality-trimmed reads were then aligned to the influenza vaccine strain reference sequence using bowtie2 (version 2.1.0) [13] with the '--very-sensitive-local' option and converted to sorted BAM files using samtools [14]. Only sequences of all 8 gene segments passed quality controls were included in the study. The sequences were submitted to NCBI GenBank database and accession numbers are listed in the Table S1.

Phylogenetics and sequence analysis

73 H1N1pdm clinical samples which yielded sufficient WGS coverage of the IAV genome were included in sequence analysis. In addition to these genomes, we retrieved two reference genomes of vaccine strains from the Global Initiative on Sharing All Influenza Data (GISAID, <https://www.gisaid.org/>) for further analyses. Nucleotide sequences of the longest open reading frames (ORFs) in each gene segment were used to build phylogenetic trees, including PB2, PB1, PA, HA, NP, NA, M1, and NS1, as well as, their alternative splicing (M2 and NS2) and ribosomal frameshift (PA-X) products. Nucleotide sequences of these ORFs (except for PA-X) in each strain were further concatenated to generate one single sequence for constructing a whole-genome phylogenetic tree. Phylogenetic trees for investigating their genetic or genomic relationships were based on maximum likelihood (ML) analysis, generated with RAxML (version 8.2.12) [15] under the GTRGAMMA model with 1000 bootstrap replicates. Their time scales based on sample collection dates were calibrated using the coalescent model in TreeTime [16] after building ML trees. Clade 6B, 6B.1, and 6B.2 were defined by the rules of HA amino acid mutations of global surveillance groups from nextflu [4]. Phylogenetic groups in other gene segments were based on their tree branches to separate annotated clades (e.g., 6B.1 and 6B.2). Inconsistent positioning of strains, or clade groups on individual trees was used to identify influenza reassortment. Individual gene segment clades were annotated and visualized using the ggtree R package [17].

All H1N1pdm genomes (n=2873) isolated from September, 2015 to May, 2016 were downloaded from GISAID as of January 2020, and were further aligned using MAFFT

tool [18] under the default conditions. Moreover, we retrieved all NP sequences (n=20,477) of H1N1pdm isolated after the year of 2009 (inclusive) from GISAID as of January 2020 to investigate the population of viruses encoding an upstream translation initiation codon (AUG) in the NP gene segment. 20,470 NP sequences were used for further analyses, after aligning and removing 7 sequences with unexpected gaps.

Glycosylation prediction

Potential N-linked glycosylation sites of the 6B.1 and 6B.2 HA and NA proteins were predicted by the NetNGlyc 1.0 server (<http://www.cbs.dtu.dk/services/NetNGlyc>).

Statistical analysis of demographic and clinical data

To identify a contribution of virus clades and genetic subgroups to clinical symptoms, we applied Chi-square or Fisher's exact tests for categorical variables and the rank-sum test for continuous variables when appropriate. The two-tailed statistical significance was set at p-value <0.05. When clinical or demographic category reached statistical significance in a univariate analysis, confounding effects were then analyzed. In addition to adjusting for possible confounding effects between demographics, disease severity and clinical outcomes, a propensity score-based weighting was applied. Briefly, we applied those potential confounders to generate the propensity (or probability) of being infected with different subgroups of H1N1pdm viruses by using machine learning based modeling methods [19]. By using the inverse probability of treatment weighting (IPTW) method, patients with higher propensity will have lower weights in the final logistic regression model to adjust for confounding factors. The weights create a pseudo-

population where the weighted virus subgroups have similar distribution across these confounders. In one simulation model, the propensity score-based weighting methods could adjust for most of the confounding effects [20]. Odds ratios of unadjusted and IPTW were calculated using logistic regression analysis.

Results:

H1N1pdm clade 6B.1 predominantly circulated in Baltimore and clade 6B.1 and 6B.2 co-circulated in northern Taiwan

From November 2015 to April 2016, 261 and 256 symptomatic patients were enrolled in the study from emergency departments in JHMI (Baltimore) and CGMH (northern Taiwan). IAV was found in 32.6% (85/261) of symptomatic patients from Baltimore and 57.8% (148/256) from northern Taiwan. About half of the IAV-positive samples were further subtyped, and the demographics and comorbidities were found to be similar. In 97.5% (39/40) of samples from Baltimore and 90.1% (64/71) of samples from northern Taiwan H1N1 was the influenza A virus subtype. The epidemic curves of the two surveillance sites were different, with influenza A virus activity starting in January and peaking in mid-March in Baltimore while IAV activity in northern Taiwan started in November and peaked in late January/early February, with peak activity higher than in Baltimore (Fink et al., MedRxiv).

Seventy-three of the 103 H1N1pdm clinical samples yielded sufficient sequence coverage of the IAV genome. Virus samples were initially analyzed based on HA segment sequences (Figure 1). In Baltimore, 91.7% (22/24) of viruses were clade 6B.1,

two were clade 6B, and no 6B.2 viruses were identified. The northern Taiwan samples consisted of 57.1% (28/49) clade 6B.1, 38.8% (19/49) clade 6B.2 and 4.1% (2/49) clade 6B viruses. The difference in clade circulation is consistent with global influenza surveillance that H1N1pdm 6B.1 and 6B.2 were dominant in the 2015-16 season and most 6B.2 viruses were found in Asia [5, 21].

Identification of four distinct genetic subgroups of H1N1pdm

To better understand the genetic diversity in the identified HA clades, we performed a phylogenetic analysis using concatenated gene segments (Figure 2) and individual ORF trees (Figure S1 and Figure 2). Branch tips of the 10 ORFs from the other seven gene segments were colored by the HA clade of each sample and defining amino acid mutations were noted (Figure S1). For the most part, the sequences of other ORFs clustered together consistent with their HA clade (Figure 2). Amino acid differences between clades 6B.1 and 6B.2 are listed for HA (Table S2), NA (Table S3) and the other ORFs (Table S4).

Additional amino acid mutations were identified to differentiate viruses into four distinct virus genetic subgroups (Figure 2). The 6B.1 viruses were separated into two groups, a 6B.1 parental and a 6B.1 variant defined by unique amino acid changes in NA and NS2 (Table S3 and S4). All Baltimore 6B.1 viruses were 6B.1 parental. In northern Taiwan, only 4 were 6B.1 parental, with the remaining clade 6B.1 viruses (23/27) being 6B.1 variant. The 6B.2 viruses were also separated into two genetic subgroups - variant-1

and variant-2 – that are differentiated by mutations in HA, NA, M1 and M2 (Table S2, S3 and S4).

Dynamic virus evolution of gene segments and possible reassortment

All segments in two of the four 6B viruses, A/EastBaltimore/0103/2016 and A/Baltimore/0088/2016, were clade 6B. One 6B virus A/Taipei/0045/2016 contained mutations in PB2, PA, NA and M segments consistent with those found in the clade 6B.1 and the remaining segments were similar but distinct from clade 6B.1. The other 6B virus A/Linkou/0030/2015 contained sequences in the PB2, M and NS segments consistent with the clade 6B.2 viruses and the remaining segments were related to but distinct from clade 6B.2. (Figure 2 and S1). These suggested that all segments of 6B viruses were evolving toward either the clade 6B.1 or clade 6B.2.

There was also evidence of intraclade reassortment in one northern Taiwan 6B.1 sample A/Keelung/0046/2016, as three gene segments of 6B.1 (segments HA, PB2 and PA) and five 6B.2 (segments PB1, NP, NA, M and NS) were detected (Figure 2). While we cannot rule out co-infection with two H1N1 clade viruses, given the number of reads present for each segment, it seems more likely this individual was infected with an intraclade reassortant virus.

HA and NA N-linked glycosylation

Eight N-linked glycosylation sites on the HA of A/California/7/2009 were previously determined by sequence analysis and verified by mass spectrometry [22]. The 6B.1 HA

sequence predicts an additional N-linked glycosylation site at 162-164 (N-Q-S) on the Sa antigenic site of the head domain due to a S to N mutation at HA-162 (Table S2) [23]. The NA from the A/California/7/2009 vaccine strain has 8 potential N-linked glycosylation sites and 6 sites were experimentally confirmed [22]. Both 6B.1 and 6B.2 viruses gain one additional predicted N-linked glycosylation site at 42-44 (N-Q-S) of the NA stalk and lose one at 386-388 (N-F-S) which lies on the lateral surface of the NA head. In addition, 68.4% (13/19) of 6B.2 viruses with NA-S52R mutation lose one glycosylation site at 50-52 (N-Q-S) on the NA stalk (Table S3).

Antiviral resistance mutations

All sequences contained the amantadine resistance mutation M2-S31N which is found in the A/Cal/07/2009 virus. One 6B.2 variant-2 sample from northern Taiwan, A/Linkou/0029/2015, contained the NA-H275Y, the most common mutation associated with oseltamivir resistance in N1 NA.

An upstream AUG codon lengthens the nucleoprotein (NP) of H1N1pdm

The NP gene segments of all samples in the study have a new, in frame AUG start codon 18 nucleotides upstream of the usual start codon of NP proteins of IAV including human seasonal H1N1 and H3N2 [24, 25], adding 6 amino acids to the amino terminus of the protein with clade 6B.1 (M-S-D-I-E-A) and 6B.2 viruses (M-S-D-I-E-V) differing at amino acid six. This start codon was shown to be utilized and the resulting NP protein capable of supporting viral polymerase activity [26, 27]. The presence of an upstream NP start codon and the amino acid difference at position 6 between 6B.1 and 6B.2 were

also present in approximately 70% of GISAID sequences (n=20,470) of H1N1pdm NP since 2009 with an Alanine (A) dominating at position 6.

Patients infected with clade 6B.2 had increased symptoms of severe influenza

To determine whether viral genotype contributes to symptoms and disease severity, the demographic and clinical data from patients infected with clade 6B.1 or 6B.2 viruses were first compared by univariate analyses (Table 1). Patients infected with 6B.2 were more likely to be older, female, Asian, and unvaccinated. These patients had a higher prevalence of runny nose and diarrhea and had increased symptoms of severe influenza, including increased likelihood of oxygen supplementation, longer in-hospital stay, and pneumonia diagnosis (by radiological findings). Interestingly, patients infected with clade 6B.1 viruses had a lower number of co-morbidities associated with severe influenza, suggesting a healthier population was being infected with those viruses.

We further utilized a logistic regression analysis to estimate the propensity of being infected by 6B.2, using potential confounders such as age, gender, comorbidities, obesity, vaccine status, human exposure and travel history (area under the ROC curve: 0.798). After IPTW to adjust for the possible confounding effects, infection with clade 6B.2 viruses was found to be significantly associated with pneumonia (Odds ratio: 3.261, 95% CI: 1.696-4.826, p=0.008) (Table 2).

The distribution of cases across the four genetic subgroups was then determined (Tables 3 and 4). Patients infected with the 6B.1 parental virus had increased reporting

of wheezing, sinus pain, and nausea/vomiting, along with the expected difference in race and ethnicity stemming from the identification of the 6B.1 variant only in northern Taiwan (Table 3). When patients infected with the 6B.2 variant-1 and variant-2 viruses were compared there were no obvious difference in symptoms (Table 4), however, the small sample sizes in the 6B.2 subgroups precluded a definitive characterization of demographic and clinical data between these genotypes. Overall, our data suggested clade 6B.2 circulation in northern Taiwan likely played a significant role in differentiating the influenza epidemics between Baltimore and northern Taiwan in the season.

Discussion:

Four distinct genetic subgroups of H1N1pdm viruses were circulating in Baltimore and northern Taiwan during the 2015-16 flu season. While the 6B.1 clade was circulating at both sites, some 6B.1 viruses in northern Taiwan had additional mutations which defined a genetic subgroup we called 6B.1 variant. The two 6B.1 subgroups of viruses did not differ drastically with respect to disease severity. The 6B.2 clade viruses were detected in northern Taiwan but not in Baltimore and consisted of two distinct genetic subgroups. Patients infected with 6B.2 virus showed more and stronger disease symptoms by both uni- and multivariate analysis. This difference in H1N1pdm clade circulation may have contributed to the more severe influenza season experienced in northern Taiwan compared to Baltimore in 2015-16. After adjusting and weighting for age, gender, comorbidities, obesity, vaccine status, human exposure and travel history, people infected with 6B.2 still had a higher odds ratio of pneumonia (Table 2). The data indicate that infection with clade 6B.2 viruses is associated with a higher disease

severity at our surveillance sites independent of patient demographics and comorbidities. The presence of the 6B.2 clade in addition to low preexisting immunity to circulating H1N1 in northern Taiwan (reference Fink, 2020 medrxiv) may be the most likely reasons explaining the different numbers and severity of influenza cases between Baltimore and northern Taiwan. The only report for symptoms of 6B.1 compared to 6B.2 infection was seen in Israel, where infection with 6B.2 virus was associated with more vomiting and nausea compared to 6B.1-infected patients [28]. In our surveillance, we did see a higher percentage of 6B.2 than 6B.1 patients with vomiting and nausea, but this did not reach statistical significance (Table 1).

The H1N1pdm 6B.1 and 6B.2 viruses dominated the 2015-16 season in the Northern Hemisphere. However, numbers of cases and disease severity differed between geographic regions. The 2015-16 H1N1pdm season in the US and Canada were moderate compared to the prior season [1, 6]. On the other hand, influenza surveillance in Russia, UK, eastern Europe, the Middle East and Asia (including Taiwan) had increasing numbers of severe cases [3, 7, 29-33]. Reports also showed H1N1pdm impacted the southern hemisphere in the 2016 season, in particular, Brazil and Reunion Island [34-36].

Serological surveillance indicated that the circulating H1N1pdm strains in 2015-16 were antigenically similar to the vaccine A/California/7/2009 in the US, UK and many other countries [1, 6, 7, 32, 37, 38]. This suggests that the HA amino acid differences between the circulating 6B.1 or 6B.2 viruses and the vaccine strain were likely not able

to explain differential epidemics in different regions of the year even though vaccination coverage might differ. Whether mutations in the other 7 viral genes contributed to the variable disease severity reported in 2015-16 has not been studied carefully. Some mutations in NA and M1 occurred at sites previously associated with altered virus transmission [39, 40], but our initial experiments on human nasal epithelial cell cultures did not detect any replication differences between clade 6B.1 and 6B.2 viruses (reference Fink, 2020 medrxiv).

We identified one 6B.2 patient in northern Taiwan had NA-H275Y mutation, which is associated with NA drug resistance. In another study of 2015-16 H1N1pdm in Taiwan, one clinical isolate with NA-H275Y was resistant to oseltamivir [41]. The data suggest sporadic identification of oseltamivir resistant H1N1pdm viruses in 2015-16 in Taiwan.

The clade 6B.1 parental genetic subgroup continued to circulate after 2015-16, and evolved into several 6B.1A subclades through August 2020. It is not clear what drove the extinction of 6B.2 but it is possible that clade 6B.1 viruses were better adapted to infect and spread in humans. It has been suggested that glycosylation is important for influenza A virus adaptation in humans [42] and gaining a potential glycosylation site on the HA head at residue 162 may have given clade 6B.1 viruses an evolutionary advantage. Clade 6B.1 viruses also encode an NS1-E125D mutation which increases NS1 interactions with cellular cleavage and polyadenylation factor 30 (CPSF30) [43]. The NS1-E125D mutation of 6B.1 viruses has been suggested to be an important marker for virus adaption to humans [7].

Our study is limited in several ways. The different race distributions between our surveillance sites make it difficult to adjust for in this and any study conducted across different geographic regions. The identification of 4 genetic subgroups reduced the power of our study to detect demographic and clinical differences in our populations. Increasing surveillance and whole genome sequencing efforts could dramatically improve the ability to identify novel virus variants that are causing altered disease in humans. Given these limitations, it is still clear that there is significant H1N1 genetic diversity across the two surveillance sites and this genetic diversity contributes to the differing number of influenza cases and disease severity. Influenza is a global disease, but surveillance at the local level is needed to fully understand virus circulation and disease potential.

Conflicts of interests

The authors declare no conflict of interest.

Author contributions

H.L., R.R., K.F.C., and A.P. conceived of the experimental questions; Y.N.G. performed phylogenetic tree analyses; K.S.S., and K.F.C. ran demographic and clinical analyses; T.M., J.E., and P.T. sequenced samples and cleaned the raw data; Z.Y.L., M.L., and L.S. oversaw enrollment and collection of samples from patients; H.L., Y.N.G., K.S.S., and K.F.C. analyzed data; H.L., and A.P. wrote the manuscript; all authors edited and reviewed the manuscript prior to submission.

Funding

This work was supported by the NIH/NIAID Center of Excellence in Influenza Research and Surveillance contract HHS N2772201400007C (R.R., K.F.C., and A.P.), the Richard Eliasberg Family Foundation and the Research Center for Emerging Viral Infections from The Featured Areas Research Center Program within the framework of the Higher Education Sprout Project by the Ministry of Education (MOE) in Taiwan and the Ministry of Science and Technology (MOST), Taiwan MOST 109-2634-F-182-001 (Y.N.G.).

Acknowledgements

The authors thank the patients who enrolled and participated in the JH-CEIRS surveillance study. We are grateful for the efforts of the clinical coordination team at JHMI who collected samples. We thank members of the Pekosz lab for feedback on this work.

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Figure Legends.

Figure 1. H1N1pdm clade 6B.1 and clade 6B.2 circulated in Baltimore and northern Taiwan in the 2015-16 season. Maximum likelihood (ML) tree of HA gene coding sequences from 73 H1N1pdm viruses of the surveillance was generated using RAxML under GTRGAMMA model with 1000 bootstrap replicates. Their time scales based on sample collection dates, noted in each sample, were calibrated using coalescent model in TreeTime after building ML tree. Reference sequences are H1N1pdm vaccine strains, A/California/07/2009 and A/Michigan/45/2015. Tips were colored by location of sample collection. Samples from Baltimore were colored in brown; samples from northern Taiwan were colored in green. Most samples were grouped into two clusters, 6B.1 (in red) and 6B.2 (in blue). Specific amino acid mutations defining major branches of 6B.1 and 6B.2 were indicated on the side of branch.

Figure 2. Four distinct genetic subgroups of H1N1pdm were identified in Baltimore and northern Taiwan in the 2015-16 season. Maximum likelihood (ML) trees of concatenated gene segments of 73 H1N1pdm viruses of the surveillance were generated using RAxML under GTRGAMMA model with 1000 bootstrap replicates. Their time scales based on sample collection dates, noted in each sample, were calibrated using coalescent model in TreeTime after building ML trees. Reference sequences are H1N1pdm vaccine strains, A/California/07/2009 and A/Michigan/45/2015. Tips were colored by location of sample collection. Samples from Baltimore were colored in brown; samples from northern Taiwan were colored in green. A schematic representation of clade/mutation of each gene segment based on their

individual gene tree branches to separate annotated clades (1, 6B, 6B.1, or 6B.2) and amino acid mutations (see Figure S1) was annotated and visualized using the ggtree R package. Mutations (Mut) in NA, M, NS, and HA segments were identified to be able to divide majority of viruses into four distinct genetic subgroups, 6B.1 parental, 6B.1 variant, 6B.2 variant-1 and 6B.2 variant-2.

Table 1. Univariate analysis of patients infected with H1N1pdm 6B.1 and 6B.2 viruses of Baltimore and northern Taiwan in the 2015-16 season

Table 2. Logistic regression analysis of pneumonia in patients infected with H1N1pdm 6B.1 and 6B.2 viruses of Baltimore and northern Taiwan in the 2015-16 season

Table 3. Univariate analysis of patients infected with H1N1pdm 6B.1 parental and 6B.1 variant viruses of Baltimore and northern Taiwan in the 2015-16 season

Table 4. Univariate analysis of patients infected with H1N1pdm 6B.2 variant-1 and 6B.2 variant-2 viruses of Baltimore and northern Taiwan in the 2015-16 season

Supplemental Figure 1. All other segments than HA of H1N1pdm viruses in the 2015-16 season were differentially evolving toward either the clade 6B.1 or 6B.2.

Phylogenetic trees of the PB2, PB1, PA, PA-X, NP, NA, M1, M2, NS1 and NS2 coding sequences (subfigures from A to J, respectively) of 73 H1N1pdm viruses of the

surveillance were generated using RAxML under GTRGAMMA model with 1000 bootstrap replicates and visualized using the ggtree R package. Branch tips were colored by HA clades, and amino acid mutations on branch were shown. Reference sequences of H1N1pdm vaccine strains, A/California/07/2009 (clade 1), and A/Michigan/45/2015 (clade 6B.1) were included. NP coding sequence numbering begins from upstream start codon.

Supplemental Table 1. GenBank accession numbers of each segment of 73 H1N1pdm strains in this study.

Supplemental Table 2. Amino acid differences (H1 numbering) of HA gene in 6B.1 and 6B.2 viruses of Baltimore and northern Taiwan in the 2015-16 season

Supplemental Table 3. Amino acid differences of NA gene in 6B.1 and 6B.2 viruses of Baltimore and northern Taiwan in the 2015-16 season

Supplemental Table 4. Amino acid differences of internal genes in 6B.1 and 6B.2 viruses of Baltimore and northern Taiwan in the 2015-16 season

Figure 1

Clade

- 6B.1
- 6B.2

Location

- Baltimore
- Northern Taiwan
- Reference

Figure 2

Clade/Mutation

A. PB2

Figure S1

C. PA

E. NP

G. M1

I. NS1

B. PB1

D. PA-X

F. NA

H. M2

J. NS2

Table 1. Univariate analysis of patients infected with H1N1pdm 6B.1 and 6B.2 viruses of Baltimore and northern Taiwan in the 2015-16 season

Characteristic		6B.1 (n=49) N (%) / median (IQR)	6B.2 (n=19) N (%) / median (IQR)	p-value
Demographics				
	Site			
	<i>JHMI</i>	22 (44.9)	0 (0)	<0.0001*
	CGMH	27 (55.1)	19 (100.0)	
	Age			
	In years	34 (28-44)	37 (32-52)	0.20 ^a
	18-49	40 (81.6)	12 (63.2)	0.12
	50-64	6 (12.2)	7 (36.8)	0.04*
	65+	3 (6.1)	0 (0)	0.55
	Gender			
	Female	31 (63.3)	14 (73.7)	0.57
	Male	18 (36.7)	5 (26.3)	
	Ethnicity			
	Non-Hispanic or Latino	47 (95.9)	19 (100.0)	0.99
	Hispanic or Latino	2 (4.1)	0 (0)	
	Race			
	Black or African American	16 (32.7)	0 (0)	<0.01*
	White	1 (2.0)	0 (0)	0.99
	Asian	27 (55.1)	19 (100.0)	<0.01*
	Other	5 (10.2)	0 (0)	0.56
	Vaccination and exposure			
	Human exposure	11 (22.4)	2 (10.5)	0.31
	Travel in last 30 days	9 (18.4)	6 (31.6)	0.33
	Influenza vaccine	8 (16.3)	0 (0)	0.09
	Co-morbidities and high risk			
	No co-morbidities	26 (53.1)	7 (36.8)	<0.01*
	One co-morbidity	15 (30.6)	8 (42.1)	0.4
	Multiple co-morbidities	8 (16.3)	4 (21.1)	0.73
	Immunosuppressive medication	1 (2.0)	0 (0)	0.99
	Pregnant	1 (2.0)	0 (0)	0.99
Disease severity				
	NEWS score	3 (0-6)	3 (0-6)	0.81 ^a
	Oxygen supplementation	2 (4.1)	3 (15.8)	0.13
	BiPAP/CPAP	1 (2.0)	0 (0)	0.99
	Intubated	0 (0)	0 (0)	0.99
	Pneumonia	7 (14.3)	7 (36.8)	0.05*
	Admitted or Observation	6 (12.2)	2 (10.5)	0.99
	LOS	7.0 (1-12)	10.0 (5-15)	0.14 ^a

	ICU	0 (0)	0 (0)	0.99
	Death	0 (0)	0 (0)	0.99
	Follow up due to sequela	3 (6.1)	1 (5.3)	0.99
	Viral co-infection	0 (0)	0 (0)	0.99
Symptoms				
	fever	47 (95.9)	19 (100.0)	0.99
	cough	48 (98.0)	19 (100.0)	0.99
	sputum	32 (65.3)	14 (73.7)	0.77
	increased sputum	17 (34.7)	6 (31.6)	0.78
	shortness of breath	35 (71.4)	10 (52.6)	0.16
	wheezing	16 (32.7)	10 (52.6)	0.16
	headache	40 (81.6)	18 (94.7)	0.26
	sore throat	39 (79.6)	15 (78.9)	0.99
	body aches	43 (87.8)	19 (100.0)	0.17
	runny nose	37 (75.5)	19 (100.0)	0.02*
	sinus pain	13 (26.5)	1 (5.3)	0.091
	fatigue	43 (87.8)	18 (94.7)	0.66
	chest pain	30 (61.2)	8 (42.1)	0.18
	chills	38 (77.6)	16 (84.2)	0.74
	loss of appetite	36 (73.5)	16 (84.2)	0.53
	nausea/vomiting	28 (57.1)	12 (63.2)	0.79
	diarrhea	10 (20.4)	9 (47.4)	0.04*
	conjunctivitis	11 (22.4)	2 (10.5)	0.33

Statistical analysis was done using R software.

Categorical variables were compared using Chi-square or Fisher's exact tests.

^a Continuous variables were compared with rank-sum test.

* Statistical significance was set at p<0.05.

Table 2. Logistic regression analysis of pneumonia in patients infected with H1N1pdm 6B.1 and 6B.2 viruses of Baltimore and northern Taiwan in the 2015-16 season

Clade	Pneumonia	Unadjusted			IPTW**		
		Odds Ratio	95% CI	p-value	Odds Ratio	95% CI	p-value
6B.1	14.3% (7/49)	1	-	-	1	-	-
6B.2	36.8% (7/19)	3.500	1.629-5.372	0.046*	3.261	1.696-4.826	0.008*

*p<0.05, logistic regression analysis.

** IPTW (inverse probability of treatment weighting) results were done by propensity score weighting with recursive partitioning algorithm (area under the ROC curve: 0.798) for age, gender, comorbidities, obesity, vaccine status, human exposure and travel history, then followed by logistic regression analysis.

Table 3. Univariate analysis of patients infected with H1N1pdm 6B.1 parental and 6B.1 variant viruses of Baltimore and northern Taiwan in the 2015-16 season

Characteristic		6B.1 Parental (n=26) N (%) / median (IQR)	6B.1 Variant (n=23) N (%) / median (IQR)	p-value
Demographics				
	Site			
	<i>JHMI</i>	22 (84.6)	0 (0)	<0.0001*
	CGMH	4 (15.4)	23 (100.0)	
	Age			
	In years	33 (29-44)	35 (29-44)	0.75 ^a
	18-49	20 (76.9)	20 (87.0)	0.47
	50-64	5 (19.2)	1 (4.3)	0.19
	65+	1 (3.8)	2 (8.7)	0.59
	Gender			
	Female	14 (53.8)	17 (73.9)	0.24
	Male	12 (46.2)	6 (26.1)	
	Ethnicity			
	Non-Hispanic or Latino	24 (92.3)	23 (100.0)	0.49
	Hispanic or Latino	2 (7.7)	0 (0)	
	Race			
	Black or African American	16 (61.5)	0 (0)	<0.0001*
	White	1 (3.8)	0 (0)	0.99
	Asian	4 (15.4)	23 (100.0)	<0.0001*
	Other	5 (19.3)	0 (0)	0.08
	Vaccination and exposure			
	Human exposure	4 (15.4)	7 (30.4)	0.36
	Travel in last 30 days	3 (11.5)	6 (26.1)	0.35
	Influenza vaccine	6 (23.1)	2 (8.7)	0.33
	Co-morbidities and high risk			
	No co-morbidities	11 (42.3)	15 (65.2)	0.15
	One co-morbidity	8 (30.8)	7 (30.4)	0.99
	Multiple co-morbidities	7 (26.9)	1 (4.3)	0.05
	Immunosuppressive medication	1 (3.8)	0 (0)	0.99
	Pregnant	1 (3.8)	0 (0)	0.99
Disease severity				
	NEWS score	3 (0-6)	3 (0-6)	0.57 ^a
	Oxygen supplementation	2 (7.7)	0 (0)	0.49
	BiPAP/CPAP	1 (3.8)	0 (0)	0.49
	Intubated	0 (0)	0 (0)	0.99
	Pneumonia	3 (11.5)	4 (17.4)	0.69
	Admitted or Observation	4 (15.4)	2 (8.7)	0.67
	LOS	6.5 (1-12)	6.0 (3-9)	0.67 ^a

	ICU	0 (0)	0 (0)	0.99
	Death	0 (0)	0 (0)	0.99
	Follow up due to sequela	2 (7.7)	1 (4.3)	0.99
	Viral co-infection	0 (0)	0 (0)	0.99
Symptoms				
	fever	24 (92.3)	23 (100.0)	0.49
	cough	25 (96.2)	23 (100.0)	0.99
	sputum	15 (57.7)	17 (73.9)	0.37
	increased sputum	10 (38.5)	7 (30.4)	0.77
	shortness of breath	20 (76.9)	15 (65.2)	0.53
	wheezing	12 (46.2)	4 (17.4)	0.04*
	headache	23 (88.5)	17 (73.9)	0.27
	sore throat	19 (73.1)	20 (87.0)	0.3
	body aches	24 (92.3)	19 (82.6)	0.4
	runny nose	17 (65.4)	20 (87.0)	0.1
	sinus pain	11 (42.3)	2 (8.7)	0.01*
	fatigue	22 (84.6)	21 (91.3)	0.67
	chest pain	19 (73.1)	11 (47.8)	0.08
	chills	21 (80.8)	17 (73.9)	0.73
	loss of appetite	20 (76.9)	16 (69.6)	0.75
	nausea/vomiting	19 (73.1)	9 (39.1)	0.02*
	diarrhea	7 (26.9)	3 (13.0)	0.3
	conjunctivitis	8 (30.8)	3 (13.0)	0.18

Statistical analysis was done using R software.

Categorical variables were compared using Chi-square or Fisher's exact tests.

^a Continuous variables were compared with rank-sum test.

* Statistical significance was set at p<0.05.

Table 4. Univariate analysis of patients infected with H1N1pdm 6B.2 variant-1 and 6B.2 variant-2 viruses of Baltimore and northern Taiwan in the 2015-16 season

Characteristic		6B.2 Variant-1 (n=6) N (%) / median (IQR)	6B.2 Variant-2 (n=13) N (%) / median (IQR)	p-value
Demographics				
	Site			
	JHMI	0 (0)	0 (0)	0.99
	CGMH	6 (100.0)	13 (100.0)	
	Age			
	In years	34 (29-48)	44 (34-54)	0.20 ^a
	18-49	4 (66.7)	8 (61.5)	0.99
	50-64	2 (33.3)	5 (38.5)	0.99
	65+	0 (0)	0 (0)	0.99
	Gender			
	Female	3 (50.0)	11 (84.6)	0.26
	Male	3 (50.0)	2 (15.4)	
	Ethnicity			
	Non-Hispanic or Latino	6 (100.0)	13 (100.0)	0.99
	Hispanic or Latino	0 (0)	0 (0)	
	Race			
	Black or African American	0 (0)	0 (0)	0.99
	White	0 (0)	0 (0)	0.99
	Asian	6 (100.0)	13 (100.0)	0.99
	Other	0 (0)	0 (0)	0.99
	Vaccination and exposure			
	Human exposure	0 (0)	2 (15.4)	0.83
	Travel in last 30 days	3 (50.0)	3 (23.1)	0.32
	Influenza vaccine	0 (0)	0 (0)	0.99
	Co-morbidities and high risk			
	No co-morbidities	3 (50.0)	4 (30.8)	0.77
	One co-morbidity	2 (33.3)	6 (46.2)	0.99
	Multiple co-morbidities	1 (16.7)	3 (23.1)	0.99
	Immunosuppressive medication	0 (0)	0 (0)	0.99
	Pregnant	0 (0)	0 (0)	0.99
Disease severity				
	NEWS score	4 (2-6)	3 (0-5)	0.54 ^a
	Oxygen supplementation	0 (0)	3 (23.1)	0.52
	BiPAP/CPAP	0 (0)	0 (0)	0.99
	Intubated	0 (0)	0 (0)	0.99
	Pneumonia	2 (33.3)	5 (38.5)	0.99
	Admitted or Observation	0 (0)	2 (15.4)	0.99
	LOS	0 (0-0)	10.0 (5-15)	<0.01*

	ICU	0 (0)	0 (0)	0.99
	Death	0 (0)	0 (0)	0.99
	Follow up due to sequela	0 (0)	1 (7.7)	0.99
	Viral co-infection	0 (0)	0 (0)	0.99
Symptoms				
	fever	6 (100.0)	13 (100.0)	0.99
	cough	6 (100.0)	13 (100.0)	0.99
	sputum	5 (83.3)	9 (69.2)	0.99
	increased sputum	3 (50.0)	3 (23.1)	0.32
	shortness of breath	2 (33.3)	8 (61.5)	0.35
	wheezing	2 (33.3)	8 (61.5)	0.35
	headache	6 (100.0)	12 (92.3)	0.99
	sore throat	6 (100.0)	9 (69.2)	0.26
	body aches	6 (100.0)	13 (100.0)	0.99
	runny nose	6 (100.0)	13 (100.0)	0.99
	sinus pain	0 (0)	1 (7.7)	0.99
	fatigue	6 (100.0)	12 (92.3)	0.99
	chest pain	2 (33.3)	6 (46.2)	0.99
	chills	5 (83.3)	11 (84.6)	0.99
	loss of appetite	6 (100.0)	10 (76.9)	0.52
	nausea/vomiting	3 (50.0)	9 (69.2)	0.62
	diarrhea	4 (66.7)	5 (38.5)	0.35
	conjunctivitis	0 (0)	2 (15.4)	0.99

Statistical analysis was done using R software.

Categorical variables were compared using Chi-square or Fisher's exact tests.

^a Continuous variables were compared with rank-sum test.

* Statistical significance was set at p<0.05.

Supplemental Table 1. GenBank accession numbers of each segment of 73 H1N1pdm strains in this study.

Strain name	HA clade	PB2	PB1	PA	HA	NP	NA	M	NS
A/Baltimore/0008/2016	6B.1	KY487410	KY487672	KY487343	KY487698	KY487491	KY487604	KY487576	KY487127
A/Baltimore/0026/2016	6B.1	KY949909	KY949764	KY950028	KY950126	KY949939	KY949753	KY950197	KY949800
A/Baltimore/0065/2016	6B.1	KY950125	KY949852	KY950143	KY950052	KY949710	KY949880	KY950094	KY950041
A/Baltimore/0076/2016	6B.1	KY487315	KY487375	KY487419	KY487689	KY487608	KY487361	KY487616	KY487333
A/Baltimore/0077/2016	6B.1	KY949732	KY949997	KY949930	KY950108	KY949912	KY949844	KY950209	KY950085
A/Baltimore/0088/2016	6B	KY487517	KY487300	KY615534	KY487140	KY487313	KY487269	KY487124	KY487551
A/Baltimore/0091/2016	6B.1	KY487652	KY487289	KY487640	KY615553	KY615415	KY487668	KY487464	KY487393
A/Baltimore/0092/2016	6B.1	KY487138	KY487194	KY615404	KY487543	KY487486	KY487463	KY487409	KY487653
A/Baltimore/0096/2016	6B.1	KY487098	KY615465	KY487452	KY615388	KY487164	KY487401	KY487428	KY487191
A/Baltimore/0101/2016	6B.1	KY487518	KY487065	KY487522	KY487503	KY487259	KY615395	KY487263	KY487762
A/Baltimore/0103/2016	6B.1	KY487208	KY487397	KY487290	KY487506	KY487341	KY487256	KY487384	KY487201
A/Baltimore/0104/2016	6B.1	KY487282	KY487502	KY487344	KY487646	KY487471	KY487475	KY487605	KY487212
A/Baltimore/0107/2016	6B.1	KY487556	KY487562	KY487722	KY487472	KY487183	KY487084	KY487451	KY487439
A/Baltimore/0110/2016	6B.1	KY487731	KY615501	KY487168	KY615408	KY487185	KY615483	KY487645	KY487752
A/Baltimore/0118/2016	6B.1	KY615383	KY615428	KY615505	KY615604	KY615376	KY615516	KY487552	KY615550
A/Baltimore/0122/2016	6B.1	KY615526	KY615507	KY487237	KY615559	KY615489	KY615491	KY487461	KY615412
A/Baltimore/0124/2016	6B.1	KY487667	KY487566	KY487101	KY487575	KY487158	KY487434	KY615563	KY487666
A/Baltimore/0125/2016	6B.1	KY487348	KY487415	KY487427	KY487453	KY487458	KY487175	KY487215	KY487497
A/Baltimore/0158/2016	6B.1	KY487156	KY615599	KY487293	KY487430	KY615592	KY487539	KY487525	KY487281
A/Baltimore/0163/2016	6B.1	KY487433	KY487770	KY487188	KY487592	KY487572	KY487523	KY615557	KY487524
A/EastBaltimore/0103/2016	6B	KY487534	KY487611	KY487704	KY487656	KY487615	KY487392	KY487465	KY487599
A/EastBaltimore/0107/2016	6B.1	KY487621	KY487721	KY487292	KY487123	KY487223	KY487136	KY487240	KY487116
A/EastBaltimore/0119/2016	6B.1	KY487252	KY487758	KY487325	KY615447	KY487381	KY487391	KY487596	KY487498
A/EastBaltimore/0141/2016	6B.1	KY615562	KY615541	KY615512	KY487527	KY487701	KY487628	KY487529	KY487696
A/Linkou/0029/2015	6B.2	KY949707	KY950139	KY949697	KY950184	KY950083	KY949706	KY950178	KY950073
A/Linkou/0030/2015	6B	KY949913	KY950117	KY950138	KY949802	KY949767	KY949760	KY949954	KY949636
A/Linkou/0032/2015	6B.1	KY949925	KY949664	KY950068	KY949823	KY950112	KY949903	KY949646	KY950070

A/Linkou/0039/2015	6B.1	KY949951	KY949669	KY949719	KY949680	KY949722	KY949743	KY949988	KY950158
A/Linkou/0046/2016	6B.1	KY949658	KY950088	KY949714	KY949679	KY949884	KY949888	KY949993	KY950131
A/Linkou/0047/2016	6B.2	KY949962	KY950065	KY949782	KY949975	KY950049	KY949999	KY949696	KY949731
A/Linkou/0049/2016	6B.1	KY950066	KY950082	KY950071	KY950223	KY949703	KY949738	KY949901	KY950106
A/Linkou/0050/2016	6B.2	KY949917	KY949704	KY949660	KY949736	KY950061	KY950170	KY949787	KY950194
A/Linkou/0057/2016	6B.1	KY487199	KY615531	KY487496	KY615390	KY615380	KY487310	KY487581	KY487584
A/Linkou/0065/2016	6B.1	KY487225	KY487445	KY487071	KY487702	KY487404	KY487488	KY487321	KY487373
A/Linkou/0068/2016	6B.1	KY487554	KY487719	KY487387	KY615570	KY487505	KY615429	KY487610	KY487555
A/Linkou/0077/2016	6B.2	KY487278	KY615572	KY487598	KY487687	KY487372	KY487383	KY487283	KY487087
A/Linkou/0084/2016	6B.2	KY487285	KY615373	KY487481	KY487767	KY487487	KY487099	KY487376	KY487500
A/Linkou/0093/2016	6B.1	KY487658	KY487319	KY487260	KY487613	KY487717	KY487623	KY487380	KY487067
A/Linkou/0097/2016	6B.1	KY487417	KY615513	KY615499	KY615425	KY615535	KY487639	KY487328	KY487213
A/Linkou/0098/2016	6B.1	KY487303	KY615539	KY487195	KY615441	KY487327	KY487137	KY487294	KY487720
A/Linkou/0099/2016	6B.1	KY487476	KY487764	KY487131	KY487353	KY487597	KY487197	KY487068	KY487072
A/Taipei/0008/2015	6B.2	KY487634	KY487510	KY487631	KY487478	KY487715	KY487143	KY487364	KY487755
A/Taipei/0015/2015	6B.1	KY487198	KY487557	KY487423	KY487273	KY615585	KY487485	KY487507	KY487200
A/Taipei/0018/2016	6B.1	KY487754	KY487607	KY487690	KY487725	KY487167	KY487501	KY487692	KY487378
A/Taipei/0021/2016	6B.1	KY487085	KY487345	KY487106	KY487147	KY615436	KY487459	KY487339	KY487211
A/Taipei/0025/2016	6B.2	KY487148	KY487326	KY487145	KY487209	KY487275	KY487484	KY487119	KY487362
A/Taipei/0030/2016	6B.1	KY615580	KY487726	KY487079	KY487336	KY487277	KY487553	KY487489	KY487386
A/Taipei/0032/2016	6B.2	KY487304	KY487371	KY487083	KY487330	KY487239	KY487718	KY487511	KY487663
A/Taipei/0037/2016	6B.2	KY487264	KY487441	KY487738	KY487649	KY487232	KY615564	KY487243	KY487349
A/Taipei/0040/2016	6B.1	KY487411	KY487538	KY615496	KY487744	KY487241	KY487229	KY487089	KY487257
A/Taipei/0045/2016	6B	KY487296	KY487456	KY487367	KY487602	KY487178	KY487714	KY487426	KY487565
A/Taipei/0046/2016	6B.1	KY487740	KY487531	KY487560	KY487436	KY487504	KY487170	KY487734	KY487635
A/Taipei/0050/2016	6B.2	KY487526	KY487474	KY487110	KY487591	KY487312	KY487102	KY487746	KY487111
A/Taipei/0054/2016	6B.1	KY487736	KY487169	KY487299	KY487403	KY487184	KY487247	KY487449	KY487258
A/Taipei/0055/2016	6B.2	KY487595	KY487249	KY487218	KY487233	KY487660	KY487733	KY487712	KY487528
A/Taipei/0059/2016	6B.2	KY487320	KY487118	KY615435	KY615586	KY615461	KY487324	KY487648	KY487291
A/Taipei/0061/2016	6B.1	KY487379	KY487546	KY487732	KY487248	KY487224	KY487350	KY487636	KY487360

A/Taipei/0063/2016	6B.2	KY487629	KY487090	KY615394	KY487365	KY487571	KY487230	KY487516	KY487174
A/Taipei/0067/2016	6B.2	KY487311	KY615407	KY487470	KY487709	KY487388	KY487675	KY487255	KY487226
A/Keelung/0011/2015	6B.1	KY487600	KY487113	KY487166	KY487251	KY487574	KY487437	KY487699	KY487593
A/Keelung/0013/2015	6B.1	KY950069	KY949665	KY949995	KY949643	KY950031	KY950092	KY949803	KY950115
A/Keelung/0027/2016	6B.2	KY487512	KY487713	KY487086	KY487340	KY487676	KY487231	KY487134	KY487418
A/Keelung/0029/2016	6B.1	KY615478	KY487334	KY615481	KY487499	KY487769	KY615400	KY487400	KY487314
A/Keelung/0031/2016	6B.2	KY487363	KY487573	KY615579	KY615480	KY487589	KY487204	KY487276	KY487444
A/Keelung/0038/2016	6B.2	KY487483	KY487739	KY487614	KY487655	KY487268	KY487466	KY487091	KY487129
A/Keelung/0039/2016	6B.1	KY949952	KY949976	KY950181	KY949887	KY949948	KY949872	KY949898	KY949946
A/Keelung/0042/2016	6B.2	KY487244	KY487186	KY487187	KY487695	KY487274	KY615567	KY487399	KY615457
A/Keelung/0046/2016	6B.1	KY487665	KY487761	KY615468	KY487149	KY487406	KY487271	KY487245	KY487217
A/Keelung/0053/2016	6B.1	KY487594	KY487609	KY487684	KY487727	KY487088	KY487181	KY487177	KY487748
A/Keelung/0055/2016	6B.1	KY949741	KY949863	KY950220	KY950148	KY949733	KY949761	KY949653	KY950042
A/Keelung/0056/2016	6B.1	KY487082	KY487495	KY615527	KY487617	KY487368	KY487416	KY487443	KY487165
A/Keelung/0065/2016	6B.2	KY487674	KY487216	KY615378	KY487219	KY487567	KY615459	KY487075	KY487579
A/Keelung/0068/2016	6B.1	KY487114	KY487742	KY487180	KY615443	KY487374	KY487728	KY487105	KY487228

Supplemental Table 2. Amino acid differences (H1 numbering) of HA gene in 6B.1 and 6B.2 viruses of Baltimore and northern Taiwan in the 2015-16 season

Vaccine/ Surveillance strains*	HA1-84	HA1-152	HA1-162	HA1-173	HA1-209	HA1-216	HA1-223	HA1-261	HA2-164	HA2-174
A/California/7/2009	S	V	S	V	T ^{&}	I	R ^{&}	A	E	D
A/Michigan/45/2015	N	V	N ^{Gly+}	V	M ^{&}	T	R ^{&}	A	E	D
6B.1 parental (n=26)	N	V	N ^{Gly+}	V	K	T	Q	A	E	D
6B.1 variant (n=23)	N	V	N ^{Gly+}	V	K	T	Q	A	E	D
6B.2 variant-1 (n=6)	S	T	S	I	K	I	Q	S	G	E
6B.2 variant-2 (n=13)	S	T	S	I	K	I	Q	A	G	E
6B.1 reassortant (n=1)	N	V	N ^{Gly+}	V	K	T	Q	A	E	D

* Twenty-six strains of 6B.1 parental group include 22 Baltimore and 4 northern Taiwan strains. 6B.1 variant, 6B.2 variant-1 and -2, and 6B.1 reassortant strains are all isolated from northern Taiwan.

^{Gly+} Amino acid changes lead to gain of potential N-linked glycosylation site.

[&] Mutations of clade 1 A/California/7/2009 vaccine X-179A or clade 6B.1 A/Michigan/45/2015 vaccine X275. The HA1 sequences of clinical isolates of A/California/7/2009 and A/Michigan/45/2015 were 209K and 223Q.

Supplemental Table 3. Amino acid differences of NA gene in 6B.1 and 6B.2 viruses of Baltimore and northern Taiwan in the 2015-16 season

Vaccine/ Surveillance strains*	NA-13	NA-34	NA-52	NA-67	NA-74	NA-96	NA-314	NA-381
A/California/7/2009	V	I	S	V	F	G	I	T
A/Michigan/45/2015	I	V	S	V	F	G	M	T
6B.1 parental (n=26)	I	V	S	V	F	G	M	T
6B.1 variant (n=23)	I	V	S	V	L	G	M	T
6B.2 variant-1 (n=6)	V	I	S	I	F	G	I	I
6B.2 variant-2 (n=13)	V	I	R ^{Gly-}	I	F	S	I	I
6B.1 reassortant (n=1)	V	I	R ^{Gly-}	I	F	S	I	I

* Twenty-six strains of 6B.1 parental group include 22 Baltimore and 4 northern Taiwan strains. 6B.1 variant, 6B.2 variant-1 and -2, and 6B.1 reassortant strains are all isolated from northern Taiwan.

^{Gly-} Amino acid changes lead to loss of potential N-linked glycosylation site.

Supplemental Table 4. Amino acid differences of internal genes in 6B.1 and 6B.2 viruses of Baltimore and northern Taiwan in the 2015-16 season

Vaccine/ Surveillance strains*	PB2- 299	PB2- 453	PA- 361	PA-X- 212	NP- 6^{&}	M1- 30	M1- 208	M2- 52	NS1- 2	NS1- 125	NS2- 2	NS2- 22	NS2- 83
A/California/7/2009	R	S	K	A	A	S	Q	Y	D	E	D	G	M
A/Michigan/45/2015	R	P	K	A	A	S	K	Y	E	D	E	G	I
6B.1 parental (n=26)	K	T	K	A	A	S	K	Y	E	D	E	G	I
6B.1 variant (n=23)	K	T	K	A	A	S	K	Y	E	D	E	V	I
6B.2 variant-1 (n=6)	R	P	R	V	V	S	Q	Y	D	E	D	G	M
6B.2 variant-2 (n=13)	R	P	R	V	V	N	Q	C	D	E	D	G	M
6B.1 reassortant (n=1)	K	T	K	A	V	N	Q	C	D	E	D	G	M

* Twenty-six strains of 6B.1 parental group include 22 Baltimore and 4 northern Taiwan strains. 6B.1 variant, 6B.2 variant-1 and -2, and 6B.1 reassortant strains are all isolated from northern Taiwan.

[&] NP coding sequence numbering begins from upstream start codon.