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Research paper

Neuraminidase inhibitor susceptibility and evolutionary analysis of human influenza B isolates from three Asian countries during 2012–2015

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ABSTRACT

Influenza B viruses of both the Yamagata and the Victoria lineages are implicated in a large proportion of the morbidity and mortality associated with influenza outbreaks. In this study, we characterized the full genomes of 53 influenza B viruses isolated during 2012–2015 in three Asian countries: Japan, Myanmar, and Vietnam. Analysis of the hemagglutinin (HA) genes revealed co-circulation of both the Yamagata and Victoria lineages within the same season in these countries. Our analysis revealed, that a large proportion of viruses circulating during 2013–2014 in Japan and Vietnam were mismatched to the vaccine supporting the rationale for using quadrivalent vaccines. Molecular analysis of the neuraminidase (NA) genes did not reveal any of the previously reported substitutions associated with reduced susceptibility to neuraminidase inhibitors (NAIs). However, one isolate from Nagasaki displayed reduced inhibition by NAIs, associated with an NA-M426I substitution (N2-numbering). Phylogenetic analysis of the eight genome segments identified a 6 + 2 reassortant strain belonging to the Victoria lineage that circulated in Japan during the 2013–2014 season. This strain appears to have evolved from a descendent of a B/Brisbane/60/2008-like strain in an intra-lineage reassortment event involving the nucleoprotein (NP) and nonstructural (NS) genes. Therefore, influenza B strains circulating worldwide continue to evolve via complex reassortment events, which contribute to their survival and the emergence of new strains. These findings highlight the need for ongoing genome-wide studies of circulating viruses and assessing the implications of these evolutionary events on the vaccines.

1. Introduction

Influenza virus belongs to the *Orthomyxoviridae* family of enveloped, segmented negative sense RNA viruses, which carries inside its nucleocapsid a genome possessing 8 segments (Nicholson et al., 2003). Influenza A and B viruses are major causes of acute respiratory infections worldwide and are associated with seasonal outbreaks (Nair et al., 2011; Tafalla et al., 2016). Influenza B viruses have been exclusively considered as human pathogens, although they were recently shown to infect seals (Fouchier et al., 2001; Bodewes et al., 2013). Influenza B

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viruses are classified based on their hemagglutinin (HA) antigens into two major, distinct lineages denoted Victoria and Yamagata lineages that were named after strains B/Victoria/2/87 and B/Yamagata/16/88, respectively (Rota et al., 1990, 1992). These strains are thought to have genetically diverged in their HA protein around 1983 and continued to evolve as distinct lineages, which occasionally exchange their genes (McCullers et al., 2004). This warrants continuous monitoring of the circulating influenza B strains.

Both influenza B lineages usually co-circulate during the influenza outbreaks (Rota et al., 1990; McCullers et al., 2004). However, it is difficult to predict which lineage will dominate in a given season. This prompted the development and introduction of quadrivalent influenza vaccines containing four strains representing the two influenza A subtypes (H3N2 and H1N1) and the two influenza B lineages (Belshe, 2010; Ambrose and Levin, 2012). Still some countries use the trivalent vaccine, which includes in addition to the A(H3N2) and A(H1N1) strains either a Yamagata-like or a Victoria-like strain.

In this study, we investigated, using full genome sequence analysis, the evolutionary dynamics of influenza B viruses from three Asian countries: Japan, Myanmar, and Vietnam and assessed their susceptibility to NAIs. We report the emergence of a 6 + 2 reassortment strain represented by the B/Niigata13F179/2014 strain.

2. Materials and methods

2.1. Ethical approval and informed consent

This study was approved by the Institutional Review Boards at all institutions were sample collection took place. An informed consent was obtained from the patient or guardian prior to sample collection.

2.2. Ethical approval and informed consent

Fifty-three human influenza B isolates collected during routine surveillance between 2012 and 2015 in Japan, Myanmar, and Vietnam were included in this study (Zaraket et al., 2016). All isolates were isolated on Madin-Darby canine kidney (MDCK) cells, a gift from H. Nishimura (Virus Research Center Sendai Medical Center Hospital, Japan).

Extraction of viral RNA was performed by using Extragen II kit (Kainos) and following the manufacturer's instructions. The extracted RNA was then processed using next-generation sequencing to determine the full genome of influenza B isolates using an Illumina MiSeq sequencer as previously described (Zaraket et al., 2016; Kanehira et al., 2015). The obtained sequence reads were trimmed and assembled by using CLC Genomics Workbench 7.0.4 (CLC bio, Inc.). The accession numbers for the influenza B sequences generated in this study are listed in Table 1.

2.3. Phylogenetic analysis

Full genome sequences of influenza B vaccine strains recommended by the WHO for the Northern and Southern hemispheres for influenza seasons 2011 to 2016 as well as other relevant human influenza B strains were downloaded from the GISAID and NCBI Influenza Virus Database websites. All gene segments were aligned using the CLUSTAL W alignment tool in MEGA 6 software (Tamura et al., 2013). Phylogenetic tree generation was performed in MEGA 6 by using a maximum likelihood approach based on the best fit model with Kimura's two parameter distance model and 1000 bootstrap replicates. Clades were designated based on the clustering of isolates in the HA phylogeny with a bootstrap support ≥70. Tree topology analysis was performed using TreeDyn software (http://www.treedyn.org/).

2.4. Glycosylation analysis

Potential glycosylation sites (i.e., amino acids Asn-X-Ser/Thr, where X is any amino acid except for Asp or Pro) in the HA and NA glycoproteins were predicted using the NetNGlyc Server 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/). A threshold value of > 0.5 for the mean potential score was considered indicative of glycosylation.

2.5. Antiviral drug susceptibility analysis

Susceptibilities of the influenza B viruses to the four NAIs: oseltamivir carboxylate (Sequoia Research, UK), zanamivir (Sequoia Research, UK), peramivir (Shionogi Co., Japan), and laninamivir (Daiichi Sankyo Co., Japan) was assessed by using a fluorescence-based NA inhibition assay with methylumbelliferone *N*-acetylneuraminic acid (MUNANA) as the substrate to calculate the 50% inhibitory concentrations (IC_{50}) of these drugs as previously described (Zaraket et al., 2016).

3. Results

3.1. Phylogenetic analysis

Complete genome sequences were obtained for 53 influenza B isolates collected during 2012–2015 in Japan, Myanmar, and Vietnam, using the Illumina Miseq platform (Table 1). The full genomes of these viruses were compared with those of the influenza B vaccine strains recommended by the WHO in the Northern and Southern hemispheres during the influenza seasons between 2011 and 2016 (Fig. 1) . According to the HA phylogeny, 23 isolates, including the Japanese viruses from the 2013/2014 season and the Vietnamese viruses from 2012 and 2013, belonged to the Victoria lineage and formed a cluster designated Vic 2. This cluster is distinct from cluster Vic 1 that harbored the vaccine strain B/Brisbane/60/2008. Vic 2 isolates possessed substitutions I146V and D197N in the 150 loop and 190 helix antigenic regions in the HA, protein respectively. A subset (n = 5) of Vic 2 isolates represented by (B/Nagasaki/13I003/2014) had an additional A202V substitution in the 190 helix antigenic epitope. The HA protein of the B/Brisbane/60/2008 strain possess 11 potential N-glycosylation sites (residues 28, 25, 145, 166, 233, 304, 333, 515, 528, 560), all of which were conserved among the isolates belonging to the Vic 2 cluster. These isolates also had a potentially additional N-glycosylated site due to the D197N substitution.

The remaining 30 isolates were Yamagata-like and were classified into a singleton (B/Vietnam/13V B-3/2013) that was designated as Yam 2 and two clusters: Yam 3 and 4. Yam 1 included B/Wisconsin/01/2010, the vaccine strain for the 2012/2013 season. Yam 3 was purely formed of Japanese isolates from the 2013/2014 season that are closely related to the vaccine strain B/Massachusetts/02/2012, the WHO recommended vaccine strain for the 2013/2014 and 2014/2015 seasons. Yam 3 isolates had only one substitution belonging to an antigenic epitope, D197N in the 190 helix relative to B/Massachusetts/02/2012. Yam 4 included a Vietnamese isolate from the 2013 season in addition to Japanese isolates from the 2013/2014 season and viruses that were isolated in Myanmar in 2014, all of which were B/Phuket/3073/2013-like, the vaccine strain for the 2015/2016 season. The Yam 4 isolates possessed 3 substitutions, S150I, N166Y, and D197N that located in three independent antigenic epitopes (the 150 and 160 loops and the 190 helix). The D197N substitution introduces a potential glycosylation site on the 190 helix epitope in addition to the 10 sites (residues 25, 59, 145, 182, 304, 347, 506, 532, 545, 577) already present in B/Massachusetts/02/2012.

We next analyzed the phylogenetic relationships of the remaining seven segments (Fig. 1). All of the genes maintained separate clades that generally corresponded to those identified in the HA tree. An exception was a subset (n = 4) of Vic-2 Japanese isolates, resembled by

Table 1
Accession numbers of influenza B gene segments sequenced in this study.

Influenza B virus	Lineage	Collection Date	Accession number							
		dd/mm/yyyy	PB2	PB1	PA	HA	NP	NA	MP	NS
B/Kyoto/13K014/2014	B(Victoria)	24/01/2014	LC032894	LC032895	LC032896	LC032897	LC032898	LC032899	LC032900	LC032901
B/Kyoto/13SK027/2014	B(Victoria)	02/04/2014	EPI566976	EPI566980	EPI566981	EPI566983	EPI566982	EPI566978	EPI566977	EPI566979
B/Kyoto/13SK043/2014	B(Victoria)	02/12/2014	EPI566984	EPI566988	EPI566989	EPI566991	EPI566990	EPI566986	EPI566985	EPI566987
B/Kyoto/13SK045/2014	B(Victoria)	13/02/2014	EPI566992	EPI566996	EPI566997	EPI566999	EPI566998	EPI566994	EPI566993	EPI566995
B/Kyoto/13SK046/2014	B(Victoria)	13/02/2014	EPI569736	EPI569735	EPI569734	EPI569729	EPI569732	EPI569731	EPI569730	EPI569733
B/Kyoto/13SK056/2014	B(Victoria)	22/02/2014	EPI569737	EPI569743	EPI569744	EPI569746	EPI569745	EPI569741	EPI569738	EPI569742
B/Kyoto/13SK056–2/2014	B(Victoria)	26/02/2014	EPI569747	EPI569751	EPI569752	EPI569754	EPI569753	EPI569749	EPI569748	EPI569750
B/Gunma/13G027/2014	B(Victoria)	18/03/2014	LC032958	LC032959	LC032960	LC032961	LC032962	LC032963	LC032964	LC032965
B/Niigata/13NU024/2014	B(Victoria)	17/4/2014	LC033070	LC033071	LC033072	LC033073	LC033074	LC033075	LC033076	LC033077
B/Niigata/13F117/2014	B(Victoria)	02/10/2014	LC033150	LC033151	LC033152	LC033153	LC033154	LC033155	LC033156	LC033157
B/Niigata/13F179/2014	B(Victoria)	17/02/2014	LC033158	LC033159	LC033160	LC033161	LC033162	LC033163	LC033164	LC033165
B/Niigata/13F290/2014	B(Victoria)	26/02/2014	LC033166	LC033167	LC033168	LC033169	LC033170	LC033171	LC033172	LC033173
B/Nagasaki/13N035/2014	B(Victoria)	29/01/2014	LC033342	LC033343	LC033344	LC033345	LC033346	LC033347	LC033348	LC033349
B/Nagasaki/13N052/2014	B(Victoria)	17/02/2014	LC033350	LC033351	LC033352	LC033353	LC033354	LC033355	LC033356	LC033357
B/Nagasaki/13N090/2014	B(Victoria)	03/06/2014	LC033358	LC033359	LC033360	LC033361	LC033362	LC033363	LC033364	LC033365
B/Nagasaki/13N092/2014	B(Victoria)	13/03/2014	LC033366	LC033367	LC033368	LC033369	LC033370	LC033371	LC033372	LC033373
B/Nagasaki/13I003/2014	B(Victoria)	28/01/2014	EPI562560	EPI562590	EPI562596	EPI562605	EPI562603	EPI562577	EPI562568	EPI562584
B/Isahaya/13I004/2014	B(Victoria)	04/03/2014	LC033390	LC033391	LC033392	LC033393	LC033394	LC033395	LC033396	LC033397
B/Vietnam/13V B-1/2012	B(Victoria)	23/11/2012	EPI580450	EPI580454	EPI580453	EPI580451	EPI580455	EPI580452	EPI580449	EPI580447
B/Vietnam/13V B-4/2013	B(Victoria)	30/08/2013	EPI566790	EPI566794	EPI566793	EPI566791	EPI566795	EPI566792	EPI566789	EPI566788
B/Vietnam/13V B-7/2013	B(Victoria)	22/08/2013	EPI566809	EPI566814	EPI566813	EPI566810	EPI566815	EPI566812	EPI566808	EPI566807
B/Vietnam/13V B-9/2013	B(Victoria)	06/11/2013	EPI566820	EPI566825	EPI566823	EPI566821	EPI566826	EPI566822	EPI566819	EPI566818
B/Okinawa/13T003/2014	B(Yamagata)	20/01/2014	EPI562608	EPI562612	EPI562611	EPI562609	EPI562613	EPI562610	EPI562607	EPI562606
B/Gunma/13G020/2014	B(Yamagata)	27/02/2014	LC032926	LC032927	LC032928	LC032929	LC032930	LC032931	LC032932	LC032933
B/Gunma/13G022/2014	B(Yamagata)	05/03/2014	LC032934	LC032935	LC032936	LC032937	LC032938	LC032939	LC032940	LC032941
B/Gunma/13G026/2014	B(Yamagata)	15/03/2014	LC032942	LC032943	LC032944	LC032945	LC032946	LC032947	LC032948	LC032949
B/Gunma/13G028/2014	B(Yamagata)	20/03/2014	LC032950	LC032951	LC032952	LC032953	LC032954	LC032955	LC032956	LC032957
B/Hokkaido/13H025/2014	B(Yamagata)	04/02/2014	LC033006	LC033007	LC033008	LC033009	LC033010	LC033011	LC033012	LC033013
B/Hokkaido/13H045/2014	B(Yamagata)	14/02/2014	LC033014	LC033015	LC033016	LC033017	LC033018	LC033019	LC033020	LC033021
B/Hokkaido/13H068/2014	B(Yamagata)	24/02/2014	LC033022	LC033023	LC033024	LC033025	LC033026	LC033027	LC033028	LC033029
B/Hokkaido/13H080/2014	B(Yamagata)	17/03/2014	LC033030	LC033031	LC033032	LC033033	LC033034	LC033035	LC033036	LC033037
B/Niigata/13NU021/2014	B(Yamagata)	27/03/2014	LC033062	LC033063	LC033064	LC033065	LC033066	LC033067	LC033068	LC033069
B/Niigata/13F001/2014	B(Yamagata)	28/01/2014	LC033174	LC033175	LC033176	LC033177	LC033178	LC033179	LC033180	LC033181
B/Niigata/13F064/2014	B(Yamagata)	05/02/2014	LC033182	LC033183	LC033184	LC033185	LC033186	LC033187	LC033188	LC033189
B/Niigata/13F180/2014	B(Yamagata)	17/02/2014	LC033190	LC033191	LC033192	LC033193	LC033194	LC033195	LC033196	LC033197
B/Niigata/13F239/2014	B(Yamagata)	24/02/2014	LC033198	LC033199	LC033200	LC033201	LC033202	LC033203	LC033204	LC033205
B/Niigata/13F336/2014	B(Yamagata)	03/03/2014	LC033206	LC033207	LC033208	LC033209	LC033210	LC033211	LC033212	LC033213
B/Niigata/13F434/2014	B(Yamagata)	11/03/2014	LC033214	LC033215	LC033216	LC033217	LC033218	LC033219	LC033220	LC033221
B/Niigata/13F498/2014	B(Yamagata)	17/03/2014	LC033222	LC033223	LC033224	LC033225	LC033226	LC033227	LC033228	LC033229
B/Nagasaki/13N070/2014	B(Yamagata)	17/02/2014	LC033374	LC033375	LC033376	LC033377	LC033378	LC033379	LC033380	LC033381
B/Nagasaki/13 N098/2014	B(Yamagata)	28/03/2014	LC033382	LC033383	LC033384	LC033385	LC033386	LC033387	LC033388	LC033389
B/Niigata/14F259/2015	B(Yamagata)	28/01/2015	EPI580539	EPI580543	EPI580542	EPI580540	EPI580544	EPI580541	EPI580538	EPI580537
B/Myanmar/14M001/2014	B(Yamagata)	06/04/2014	EPI568108	EPI568112	EPI568111	EPI568109	EPI568113	EPI568110	EPI568107	EPI568106
B/Myanmar/14M101/2014	B(Yamagata)	28/07/2014	EPI568116	EPI568120	EPI568119	EPI568117	EPI568121	EPI568118	EPI568115	EPI568114
B/Myanmar/14M138/2014	B(Yamagata)	13/08/2014	EPI568124	EPI568128	EPI568127	EPI568125	EPI568129	EPI568126	EPI568123	EPI568122
B/Myanmar/14M225/2014	B(Yamagata)	28/07/2014	EPI568132	EPI568136	EPI568135	EPI568133	EPI568137	EPI568134	EPI568131	EPI568130
B/Myanmar/14M238/2014	B(Yamagata)	03/08/2014	EPI568140	EPI568144	EPI568143	EPI568141	EPI568145	EPI568142	EPI568139	EPI568138
B/Myanmar/14M297/2014	B(Yamagata)	25/07/2014	EPI568148	EPI568152	EPI568151	EPI568149	EPI568153	EPI568150	EPI568147	EPI568146
B/Myanmar/14M408/2014	B(Yamagata)	24/08/2014	EPI568156	EPI585080	EPI568159	EPI568157	EPI568161	EPI568158	EPI568155	EPI568154
B/Myanmar/14M456/2014	B(Yamagata)	05/09/2014	EPI568164	EPI568168	EPI568167	EPI568165	EPI568169	EPI568166	EPI568163	EPI568162
B/Vietnam/13V B-3/2013	B(Yamagata)	01/03/2013	EPI566782	EPI566786	EPI566785	EPI566783	EPI566787	EPI566784	EPI566781	EPI566780
B/Vietnam/13V B-6/2013	B(Yamagata)	13/08/2013	EPI566799	EPI573632	EPI566803	EPI566801	EPI566805	EPI566802	EPI566798	EPI566797

the B/Niigata/13F117/2014, from the 2013/2014 season which departed from the Vic 2 cluster to form an independent cluster in the NP and NS trees. The B/Niigata/13F117/2014-like cluster was more closely related to the Yam1 cluster on the NP tree but was divergent from the other clusters on the NS tree. The four isolates belonging to the B/Niigata/13F117/2014-like cluster possessed identical NS genes, suggesting that they are derived via a reassortment event from a common and recent ancestor. The fact that some of the strains that were closely related to the B/Niigata/13F117/2014-like isolates on the HA tree (Vic 2 cluster) did not group with this strain in the NP and NS trees also support the recent history of this reassortment event.

Overall, these findings suggest that the B/Niigata/13F117/2014-like strain has emerged from a recent 6 + 2 intra-lineage reassortment event involving the exchange of the NP and NS segments from another strain of an unknown origin. To better understand the origin of the B/Niigata/13F117/2014 strain, we blasted its NP sequence and included the most closely related isolates that were returned by the search and for which the full genomes were available. In both the NP and NS trees, these sequences formed a cluster that was closely related to the B/Niigata/13F117/2014-like strain. Overall, these sequences clustered within B/Brisbane/60/2008-like strain cluster or Vic 1 in all of the segment trees except for the NS tree. In the NS tree, most of these strains detached from Vic 1 cluster and were closely related to the B/Niigata/13F117/2014-like strain. These findings indicate that the B/Niigata/13F117/2014-like strain might have acquired its NP and NS genes from a descendent of a B/Brisbane/60/2008-like strain. To confirm these findings a heat map was generated using the pairwise distance values relative to B/Brisbane/60/2008 (Fig. 2). The heat map revealed a sudden shift in the similarity scores of the NP and NS genes of the B/Niigata/13F117/2014-like isolates towards B/Brisbane/60/

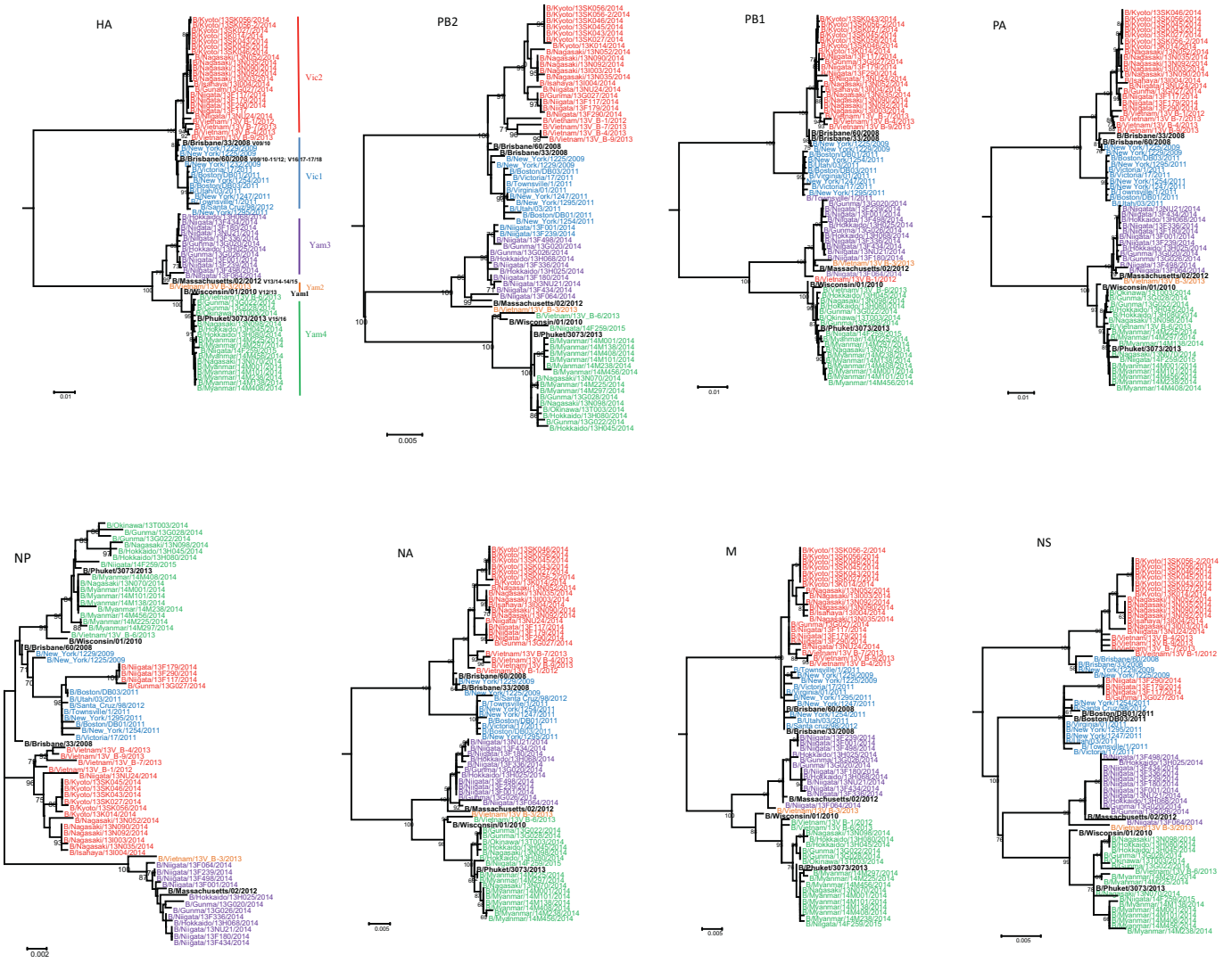

Fig. 1. Phylogenetic tree analysis of the eight genome segments of human influenza B viruses from Japan, Myanmar, and Vietnam. The phylogenetic tree for each genome segment was constructed using maximum likelihood analysis based on the best-fit nucleotide substitution model for each segment. The Hasegawa-Kishino-Yano model with gamma distribution was used for the HA, PB2, PA, NP, PA, M, and NS genes, while the Tamura-Nei and T92: Tamura 3-parameter with Gamma distributions were used for the PB1 and NA, respectively. Bootstrap support values ≥70%, which corresponds to a ≥ 95% probability that a given clade is real, are shown. Whole genome sequences of the WHO-recommended vaccine strains for the seasons covered by the study, obtained from the Influenza Resource Database, were included in the analysis for comparison purposes. The vaccine strains are indicated in black font. The season(s) for which these vaccine strains were recommended are indicated in the HA tree as follows: e.g. V09/10 for season 2009/2010, V11/12 for season 2011/2012, etc.

2008, indicating a common ancestor.

3.2. Analysis of the genetic markers in the NA gene conferring resistance to NAIs

Three neuraminidase inhibitors (NAIs), oseltamivir zanamivir, and peramivir, are currently available for the prophylaxis and treatment of influenza B viruses. In addition, laninamivir is approved in Japan for prophylaxis and treatment (Zaraket and Saito, 2016). The emergence of NAI-resistant influenza B strains is a public health concern. The catalytic site of the NA enzyme contains 19 highly conserved amino acids. These catalytic residues include (R118, D151, R152, R224, E276, R292, R371, Y406; N2 numbering here and throughout the text) that have direct interaction with sialic acid, and framework residues (E119, R156, W178, S179, D/N198, I222, E227, H274, E277, N294, E425) that support the catalytic residues for functional binding and catalysis (Burnham et al., 2013; Burmeister et al., 1992; Colman et al., 1983; Colman et al., 1993). Substitutions at these conserved sites confer resistance to NAIs. None of the 53 influenza B viruses that were analyzed in this study displayed any substitutions in the conserved residues of the NA active site or in the framework residues.

Several amino acid substitutions in the NA protein have been associated with reduced susceptibility of influenza B viruses to NAIs (Burnham et al., 2013; WHO, 2016). None of the 53 influenza B samples possessed any of the amino acid substitutions recommended for monitoring in the NA protein. One isolate, B/Kyoto/13 K014/2014 possessed an A395V substitution in the NA. This substitution was recently shown to enhance the NA activity without affecting its susceptibility to NAIs (Tewawong et al., 2018). Whereas, an A395E substitution has been associated with reduced susceptibility to both oseltamivir and peramivir (Leang et al., 2014). Regarding N-glycosylation, our analysis predicted 4 potential sites (residues 56, 64, 144, and 284) that were conserved among all the influenza B isolates including the vaccine strains.

3.2.1. Phenotypic susceptibility to NAIs

We next assessed the IC_{50}s of the four NAIs against a subset (n = 17) of influenza B isolates and compared them with the values of the

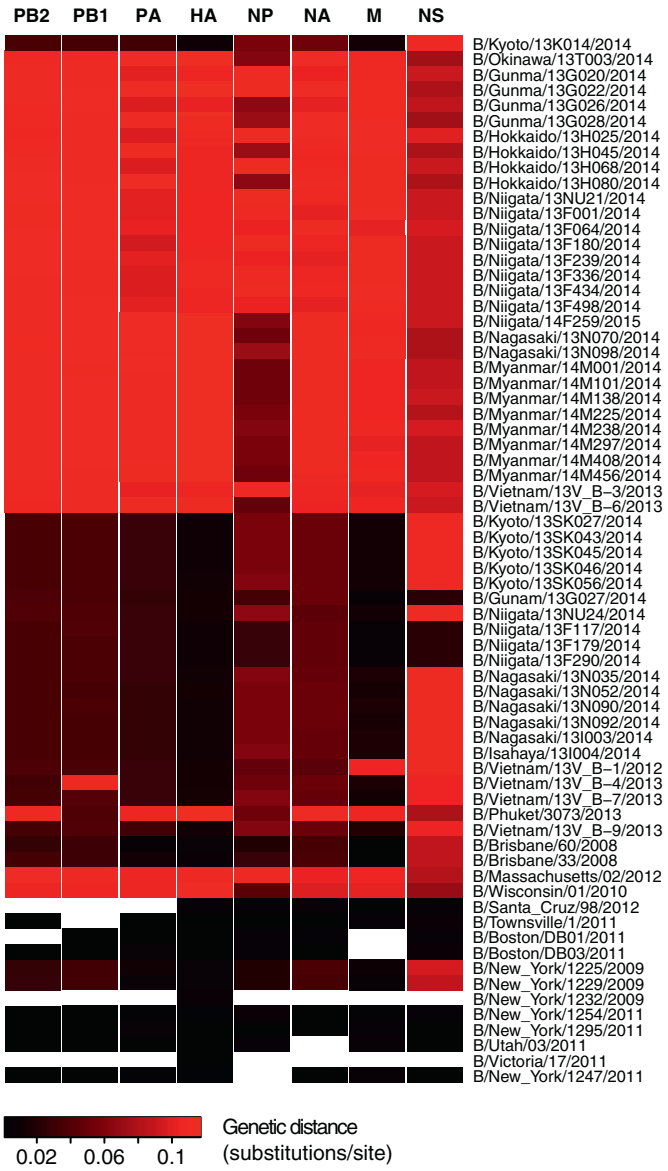

Fig. 2. Heat map of the pairwise distance values. A representation of the pairwise distance of each of the influenza B genome segments relative to those of the B/Brisbane/60/2008 vaccine strain.

reference, susceptible strain (B/PERTH/211/2001). The influenza antiviral susceptibility (AVWG) of the Global Influenza Surveillance and Response System (GISRS) has defined antiviral susceptibility of influenza B viruses based on the fold change of the IC_{50} value compared to the reference strain as follows: normal (< 5-fold), reduced (5–50-fold) and highly reduced (> 50-fold) inhibition (World Health Organization, 2012). The average IC_{50}s were 27.4 ± 10.9 nM for oseltamivir, 21.9 ± 12.8 nM for zanamivir, 1.2 ± 0.8 nM for peramivir, and 7.6 ± 4.7 nM for laninamivir (Table 2). The B/Kyoto/13 K014/2014 isolate possessing the A395V substitution had normal inhibition by the four NAIs (IC_{50} = 27.2, 12.5, 1.1, and 4.1 nM for oseltamivir, zanamivir, peramivir, and laninamivir, respectively, consistent with the recent findings by Tewawong et al. (Tewawong et al., 2018). One isolate, B/Nagasaki/13 N052/2014, displayed reduced inhibition by peramivir (8.4-fold the reference values) and laninamivir (7-fold) but normal, although slightly elevated (~3.5-fold the reference values), IC_{50s} for oseltamivir and peramivir. This isolate has two unique substitutions (D320N and M426I) compared to the rest of the isolates. Residue 426 is near the framework residue E425, which locates beneath the catalytic and framework residues R118 and E119, respectively (Fig. 3). A subset (n = 6) of the isolates from Tokyo, represented by B/Kyoto/13SK027/2014 and belonging to the Vic2 cluster, had slightly higher IC_{50} values for zanamivir and laninamivir relative to the susceptible reference strain, but they were all classified as normal inhibition according to the AVWG criteria (Table 2). These isolates possessed a unique G248D substitution that lies on top of the framework residue H274, which in turn neighbors the catalytic residue E277 (Fig. 3). A bulkier aspartic acid (D) at residue 248, which points towards residue 274 might push this site inwards and destabilize the interaction between the binding site and the inhibitor. Furthermore, the average IC_{50} values of the four NAIs for the selected viruses of both the B/Victoria and B/Yamagata lineages were compared. The average IC_{50} of the B/Victoria-like NA was 1.3 to 1.9-fold higher than that of the B/Yamagata-like NA across all the tested NAIs.

4. Discussion

In this study, we analyzed the complete genomes of influenza B viruses that were isolated in three Asian countries, Japan, Myanmar, and Vietnam, between 2012 through 2015. Our data shows that the HA protein of our isolates diversified into one Victoria cluster and two Yamagata clusters and a singlet that also belonged to the Yamagata lineage. Notably, influenza B isolates belonging to the Victoria lineage and multiple Yamagata strains co-circulated in Japan and Vietnam during the same period. While, in Myanmar only one Victoria strain circulated. During the 2013/2014 season, a Yamagata lineage strain (B/Massachusetts/02/2012) was the recommended vaccine strain in Japan and Vietnam; however, a large proportion of the isolates from these countries belonged to the Victoria lineage. This strongly supports the recommendation to use quadrivalent influenza vaccines, which include both Victoria- and Yamagata-like strains in addition to the A(H1N1) pdm09 and A(H3N2) viruses (Belshe, 2010; Ray et al., 2017).

In this study, the Japanese isolates from the 2013/2014 season belonged to two distinct Yamagata clusters (designated Yam 3 and Yam 4). Yam 3 isolates had identical antigenic epitopes as in the B/Massachusetts/02/2012, except for one substitution, D197N in the 190 helix antigenic epitope in the HA, which introduces a potential N-glycosylation at this residue. This substitution was previously detected among influenza B isolates detected in Italy and Thailand between 2010 and 2014 (Tramuto et al., 2016; Horthongkham et al., 2016). The Yam 4 isolates possessed two additional substitutions (S150I and N166Y) in the antigenic epitopes, 150 and 160 loops, previously reported in Italy and Thailand during the same period (Tramuto et al., 2016; Horthongkham et al., 2016). Interestingly, the Yam 4 isolates were closely related to the B/Phuket/3073/2013 strain that possessed both the S150I and N166Y substitutions but not the D197N substitution. However, the B/Phuket/3073/2013 strain was not designated as the vaccine strain until the 2015/2016 season.

NAIs provide important means for prophylaxis and treatment of influenza infections including those caused by influenza B viruses (Burnham et al., 2013). Several amino acid substitutions that reduce influenza B susceptibility to NAIs have emerged (Burnham et al., 2013; WHO, 2016). Some of these substitutions were demonstrated to have a competitive advantage over the wild-type sensitive viruses (Burnham et al., 2015). Between 2012 through 2015, the prevalence of influenza B viruses with reduced susceptibility to NAIs ranged between 0.7 and 2% among B/Victoria strains and 0.3–1% among B/Yamagata viruses (Meijer et al., 2014; Takashita et al., 2015; Hurt et al., 2016). In this study, none of the analyzed influenza B viruses possessed any of the substitutions reported to reduce its susceptibility to NAIs and all tested viruses had normal inhibition by the NAIs. One isolate from Nagasaki (B/Nagasaki/13 N052/2014) in Japan had reduced inhibition by peramivir and laninamivir and slightly elevated IC_{50} values for oseltamivir and zanamivir. This isolate possessed a unique M426I substitution close to a framework residue. A substitution at this site might affect the

Table 2
Susceptibility of influenza B viruses to NAIs in fluorescence- based phenotypic assay.

Influenza B virus	Lineage	IC_{50} (nM)			
		Oseltamivir	Zanamivir	Peramivir	Laninamivir
B/Kyoto/13K014/2014	B(Victoria)	27.2	12.5	1.1	4.1
B/Kyoto/13SK027/2014	B(Victoria)	28.0	40.9	1.1	13.6
B/Kyoto/13SK043/2014	B(Victoria)	28.9	31.6	1.0	12.2
B/Kyoto/13SK045/2014	B(Victoria)	28.6	30.7	1.1	12.3
B/Kyoto/13SK046/2014	B(Victoria)	28.1	33.2	1.1	12.2
B/Kyoto/13SK056/2014	B(Victoria)	28.7	33.5	1.0	15.1
B/Kyoto/13SK056–2/2014	B(Victoria)	23.2	30.4	1.1	9.4
B/Niigata/13NU024/2014	B(Victoria)	22.8	15.7	1.0	5.4
B/Nagasaki/13N052/2014	B(Victoria)	71.0	58.5	4.2	20.3
B/Nagasaki/13I003/2014	B(Victoria)	28.8	11.5	1.1	4.4
Average ± SD		31.53 ± 14.05	29.85 ± 14.18	1.38 ± 0.99	10.9 ± 5.16
B/Okinawa/13T003/2014	B(Yamagata)	31.4	30.6	2.6	6.0
B/Hokkaido/13H045/2014	B(Yamagata)	13.9	18.9	0.4	3.9
B/Niigata/13NU021/2014	B(Yamagata)	18.8	9.3	0.7	3.8
B/Niigata/13F180/2014	B(Yamagata)	18.7	11.8	0.6	4.8
B/Myanmar/14M001/2014	B(Yamagata)	22.7	13.2	0.9	4.4
B/Myanmar/14M101/2014	B(Yamagata)	24.4	10.4	0.8	4.5
B/Myanmar/14M138/2014	B(Yamagata)	21.9	14.8	0.8	5.3
B/Myanmar/14M225/2014	B(Yamagata)	22.3	11.6	0.8	3.6
B/Myanmar/14M238/2014	B(Yamagata)	37.3	25.9	1.2	8.1
B/Myanmar/14M297/2014	B(Yamagata)	25.6	12.4	0.8	4.5
B/Myanmar/14M408/2014	B(Yamagata)	25.5	11.3	0.9	4.4
B/Myanmar/14M456/2014	B(Yamagata)	24.9	12.3	0.9	4.7
Average ± SD		23.95 ± 6.06	15.21 ± 6.63	0.95 ± 0.55	4.83 ± 1.22
Overall Average ± SD		27.3 ± 10.9	21.9 ± 12.8	1.2 ± 0.8	7.6 ± 4.7
B/PERTH/211/2001[a]		19.3 ± 2.7	15.2 ± 1	0.5 ± 0.03	2.9 ± 0.3

[a] Reference susceptible strain; values are average ± SD of triplicates.

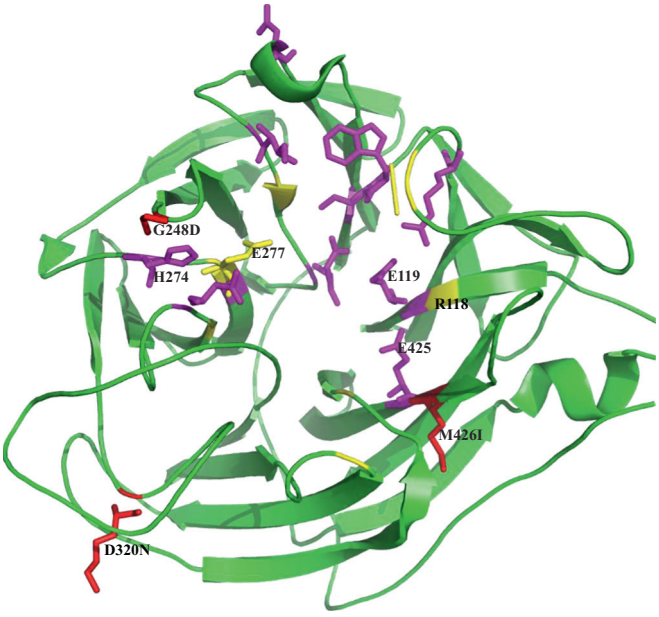

Fig. 3. Structural representation of the influenza B virus NA substitutions associated with altered susceptibility to NAIs. Cartoon rendering of influenza B virus NA protein (pdb 1VCJ) with key residues drawn in sticks. Catalytic residues are colored in yellow, framework residues are in magenta, and substitutions associated with altered susceptibility are in red. 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.)

conformation of the neighboring framework residue and the catalytic and framework residues with which it interacts that could weaken the interaction of the inhibitor with the substrate binding residues. Notably, the IC_{50}s of NAIs against the B/Victoria-like isolates were slightly higher than those belonging to the B/Yamagata lineage. Consistent with our observations, a worldwide survey in 2011 also reported an approximated 2-fold increase in the mean IC_{50} of B/Victoria compared to B/Yamagata for 3 different NAIs (Okomo-Adhiambo et al., 2013). Farrukee et al. reported that some substitutions occurring in the framework and monomeric interface residues of the influenza B NA result in higher IC_{50} values in the B/Victoria compared to the B/Yamagata viruses, albeit the difference is typically < 10-fold (Farrukee et al., 2015). Therefore, it can be concluded that B/Victoria strains are naturally less susceptible to NAIs compared to the B/Yamagata lineage.

Inter-lineage and intra-lineage or -clade reassortment events as well as antigenic drifts due to point substitutions in the HA protein can contribute to the emergence of new strains and result in global outbreaks (Oong et al., 2015; Dudas et al., 2014; Oong et al., 2017; Pan et al., 2015). Of the eight genome segments, only the PB2, PB1 and HA genes still survive as separate Victoria and Yamagata lineages. In contrast, the PA, NP, NA and MP genes of recent Victoria and Yamagata strains are all derived from Yamagata, while the NS segment is completely derived from the Victoria lineage (Dudas et al., 2014). In this study, influenza B/Niigata/13F117/2014 virus demonstrated incongruent topologies in the NP and NS trees with respect to the rest of genes revealing a recent reassortment event. Evolutionary analysis suggested that the B/Niigata/13F117/2014-like reassortant strain might have acquired its NP and NS genes from an undetermined descendent of a B/Brisbane/60/2008-like strain in an intra-clade reassortment event.

Molecular characterization of influenza B viruses isolated in other Asian countries have also revealed various reassortment events that took place during the past seasons. In Malaysia, B/Phuket/3073/2013-like viruses isolated during 2013 and 2014 were found to have evolved from intra-clade and inter-clade reassortment events within the Yamagata lineage (Oong et al., 2015), This intra-clade reassortant B/Phuket/3073/2013-like strain predominated during 2014 in Malaysia (Oong et al., 2017). The genetic composition of Malaysian B/Phuket/3073/2013-like viruses were distinct from the WHO-recommended vaccine strains for the Southern hemisphere during 2012 through 2016

(Oong et al., 2015). In Beijing, China, a subset of influenza B isolates from SARI (severe acute respiratory illness) cases possessed HA of Yamagata background and NA of Victoria origin, revealing an intra-lineage reassortment background (Pan et al., 2015). Inter-lineage reassortant influenza B strains were also reported in India in 2010 (Patil et al., 2013). In general, these studies highlight the complex evolution of influenza B isolates which involves within and cross-lineage reassortment events.

Continued surveillance of influenza B viruses and characterization of their full genomes is essential to monitor their evolutionary dynamics over time. It will be interesting to observe the evolution of influenza B viruses in response to vaccination as the implementation of quadrivalent vaccine, which includes both the Yamagata and Victoria strains, increases globally.

Declaration of interest

The authors declare no conflicts of interest.

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