博士論文

Characterization of currently circulating influenza B viruses

(B 型インフルエンザウイルス流行株の性状解析)

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CONTENTS

PREFACE

Infectious diseases have had devastating effects on the human population throughout history. Due to the discovery of antibiotics and the development of vaccines against pathogens, the prevention and treatment of infectious diseases has progressed extensively. For example, smallpox was eradicated due to an intensive eradication program led by the World Health Organization (WHO).[1] Many factors contributed to the elimination of smallpox, but vaccination played a particularly crucial role. Yet, aside from smallpox, the efficient prevention or treatment of most infectious diseases, such as AIDS, Ebola, Cholera, and influenza, has not been achieved. Therefore, researchers around the world have been trying to develop better prevention or therapeutic options for these infectious diseases.

Influenza viruses, which belong to the *Orthomyxoviridae* family, possess single-stranded, negative-sense RNA genomes. These viruses are classified into four types (types A, B, C, and D) on the basis of the antigenicity of their internal proteins, nucleoprotein (NP), and matrix protein (M1).[2,3] Influenza A and B viruses are responsible for annual seasonal epidemics in humans. Globally, each year, an estimated 5%–10% of adults and 20%–30% of children are infected with seasonal influenza viruses, causing 3–5 million cases of severe illness,[4] and an estimated 290,000–650,000 influenza-related deaths.[5] Therefore, seasonal influenza is associated with significant morbidity and mortality worldwide. Influenza A viruses are further divided into subtypes based on the antigenicity of two surface glycoproteins, hemagglutinin

(HA, H1-H18) and neuraminidase (NA, N1-N11). Although influenza B viruses are not classified into subtypes, since 1983 they have circulated as two distinct lineages: the B/Yamagata/16/88-like (B/Yamagata)- and B/Victoria/2/87-like (B/Victoria)-lineages.[6,7] Currently, two subtypes of influenza A viruses (A/H1N1 and A/H3N2) and the two lineages of influenza B viruses are cocirculating in the human population.

Influenza B virus infection is often perceived to be milder than influenza A virus infection. However, a recent study showed that influenza B virus caused similar clinical characteristics and outcomes as influenza A viruses among adults hospitalized with seasonal influenza infection.[8] In addition, a cohort study in Canada reported that among hospitalized children, influenza B virus infections resulted in greater mortality than influenza A virus infections.[9] These findings suggest that influenza B viruses can pose a significant disease burden during seasonal influenza epidemics.[10] Nevertheless, influenza B viruses have not been as well studied and characterized as influenza A viruses.[11] Therefore, the aim of this study was to elucidate the virological characteristics of the currently circulating influenza B viruses.

ABSTRACT

Influenza B viruses are responsible for severe morbidity and mortality worldwide during seasonal influenza epidemics. In this study, I genetically and antigenically analyzed influenza B viruses isolated in Japan during the 2017–2018 and 2018–2019 influenza seasons. A total of 68 influenza B viruses (61 B/Yamagata-lineage and 7 B/Victoria-lineage) were recovered from respiratory specimens collected from patients in Japan during these two seasons. influenza B virus isolates were antigenically and genetically characterized by using hemagglutination inhibition assays and phylogenetic analysis, respectively. All 61 B/Yamagata-lineage isolates were genetically closely related to B/Phuket/3073/2013, the vaccine strain for these two seasons. Eleven B/Yamagata-lineage isolates tested were antigenically similar to B/Phuket/3073/2013. Seven B/Victoria-lineage isolates were genetically closely related to B/Texas/02/2013, the vaccine strain for the 2017–2018 season; however, they were antigenically distinct from B/Texas/02/2013. Of these 7 isolates, 4 possessed a two-amino-acid deletion at positions 162 and 163 in HA and the other 3 had a three-amino-acid deletion at positions 162–164 in HA. Importantly, the variants with the three-amino-acid deletion appeared to be antigenically different from the B/Colorado/06/2017 virus with the two-amino-acid deletion, the vaccine strain for the 2018–2019 season. One B/Yamagata-lineage isolate carrying a G407S mutation in its NA, which confers reduced susceptibility to NA inhibitors, was detected. These results highlight the need for continued monitoring for the prevalence of the

antigenic variant with the three-amino-acid deletion and the variant with reduced NA inhibitor susceptibility.

INTRODUCTION

Influenza B viruses cause epidemics of influenza worldwide, mainly during the winter season. The influenza B viruses possess two major surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA). HA is the major antigen, which elicits the production of neutralizing antibodies by the host upon infection or vaccination.[12] The accumulation of point mutations in the antigenic sites of HA enables viruses to evade host immune responses induced by prior infections or vaccinations, resulting in the emergence of new antigenic variants with epidemic potential.[13,14] Anti-influenza drugs that inhibit the enzymatic activity of NA are available for the treatment and prophylaxis of influenza; however, mutations in the NA active site reduce its susceptibility to NA inhibitor drugs, leading to the emergence of drug-resistant variants.[15,16] Therefore, the characterization of circulating influenza B viruses is essential to assess and monitor the emergence of new antigenic variants and drug-resistant variants.

During the 2017–2018 influenza season, influenza A/H1N1 2009 pandemic (A/H1N1pdm), A/H3N2, and B viruses co-circulated in Japan. Notably, in Japan, the influenza B epidemic of this season was larger than that of the previous nine seasons. In this study, I examined the genetic and antigenic properties of the influenza B viruses isolated in Japan during the winter season. In addition, I characterized the influenza B viruses isolated in Japan during the 2018–2019 season, even though influenza B viruses circulated at a lower level in this season compared with the previous season.

MATERIALS AND METHODS

Cells. Madin-Darby canine kidney (MDCK) and AX4[17] cells were maintained in Eagle's minimal essential media (MEM) containing 5% newborn calf serum (NCS). hCK[18] cells were maintained in the presence of 2 μg/ml puromycin and 10 μg/ml blasticidin in MEM containing 5% NCS. Human embryonic kidney 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum. The cells were incubated at 37 °C with 5% CO_2, and regularly tested for mycoplasma contamination by using PCR and were confirmed to be mycoplasma-free.

Antiviral compounds. Oseltamivir carboxylate, peramivir, and laninamivir were kindly provided by Daiichi Sankyo Inc, Tokyo, Japan. Zanamivir was obtained from GlaxoSmithKline, London, UK.

Clinical specimens. After informed consent was obtained, respiratory specimens were collected from patients with influenza-like symptoms who visited clinics in Tokyo, Japan during the 2017–2018 and 2018–2019 seasons. For pediatric patients, informed consent was obtained from the parents. The specimens were submitted to the Division of Virology, Department of Microbiology and Immunology, Institute of Medical Science, the University of Tokyo for virus isolation. The research protocol was approved by the Research Ethics Review

Committee of the Institute of Medical Science of the University of Tokyo (approval no. 26-42-0822). Samples that were influenza B-positive by real-time RT-PCR (see below) were used in this study.

Viruses. Influenza B viruses were propagated in MDCK, AX4, or hCK cells in MEM containing 1 μg of *L*-1-*Tosylamide-2-phenylethyl chloromethyl ketone* (*TPCK*)-trypsin/ml at 33 °C.

Reverse genetics. Plasmid-based reverse genetics for influenza virus generation was performed as previously described.[19] In brief, plasmids encoding the complementary DNAs for the HA and NA genes derived from B/Phuket/3073/2013, and PB2, PB1, PA, NP, M and NS genes derived from B/Yamagata/1/73[20] under the control of the human RNA polymerase I promoter and the mouse RNA polymerase I terminator (referred to as PolI plasmids), and plasmids for the expression of the viral PB2, PB1, PA, and nucleoprotein proteins derived from influenza B virus strain B/Yamagata/1/73, under the control of the chicken β-actin promoter,[21] were transfected into 293T cells with the help of a transfection reagent, Trans-IT 293 (Mirus). At 48 h post-transfection, culture supernatants were collected and inoculated to MDCK cells for virus propagation. All virus stocks were sequenced to confirm the absence of unwanted mutations.

Real-time RT-PCR. RNA was extracted from clinical specimens by using the simplyRNA Tissue Kit (Promega) or RNeasy Mini Kit (Qiagen). Amplification and detection by real-time PCR were performed with the Applied Biosystems 7900HT Fast Real-Time PCR System (Applied Biosystems), the StepOnePlus Real-Time PCR System (Applied Biosystems), or the LightCycler 96 System (Roche). RT-PCR was carried out using the QuantiTect multiplex RT-PCR kit (Qiagen) or QuantiTect Probe RT-PCR Kit (Qiagen). The probes contained oligonucleotides with the 6-carboxyfluorescein (FAM) or the hexacholoro-6-carboxyfluorescein (HEX) reporter dye at the 5′ end, and the Black Hole Quencher-1 (BHQ-1) or 6-carboxytetramethylrhodamine (TAMRA) quencher dye at the 3′ end. A list of the primers and probes used is provided in **Table 1**.

Virus isolation. MDCK, AX4, or hCK cells grown in 12-well plates were inoculated with 0.1 ml per well of the clinical samples and incubated at 33 °C for at least 30 min. One milliliter of MEM containing 0.3% bovine serum albumin (BSA) and 1 μg/ml TPCK-treated trypsin was then added to cells. The cultures were then incubated for up to 7 days, until cytopathic effects were evident. Cell culture supernatants were harvested, viral RNA was extracted and subjected to RT-PCR, and the viral genes were sequenced (see below).

RT-PCR and sequencing of viral genes. Viral RNA was extracted by using the QIAmp Viral

RNA Mini Kit (Qiagen) and reverse-transcribed to cDNA by using Superscript III reverse transcriptase (Thermo Fisher Scientific) and the Uni9/FluB1 primer. PCR was performed with primers specific for the HA or NA genes of influenza B virus. The PCR products were purified and directly sequenced by using BigDye Terminator version 3.1 Cycle Sequencing Kits (Thermo Fisher Scientific), and were then analyzed on an ABI Prism 3130xl Genetic Analyzer (Thermo Fisher Scientific). A list of the primers and probes used is provided in **Table 1**.

Phylogenic analysis. Nucleotide sequences of the HA genes of influenza B viruses were aligned using MEGA version 7.0.26.[22] The phylogenic tree of the HA nucleotide sequences was built in maximum-likelihood method with 1,000 bootstrap replicates using MEGA version 7.0.26.

Experimental infection of ferrets. Six- to seven-month-old female ferrets (Wuxi Sangosho Biotechnology Co., Ltd.) were used in this study. Ferrets were anesthetized intramuscularly with ketamine and xylazine (5–30 mg and 0.2–6 mg/kg of body weight, respectively), and inoculated intranasally with 10^6 PFU (0.5 ml) of B/Yokohama/5/2004, B/Brisbane/60/2008, B/Wisconsin/01/2010, B/Massachusetts/02/2012, B/Phuket/3073/2013, B/Texas/02/2013, or B/Colorado/06/2017. Serum samples were collected and subjected to the hemagglutination inhibition (HI) assay. All experiments with ferrets were performed in accordance with the

University of Tokyo's Regulations for Animal Care and Use and were approved by the Animal Experiment Committee of the Institute of Medical Science, the University of Tokyo.

HI assay. Ferret sera were treated with receptor-destroying enzyme (RDE II; Denka Seiken Co., Ltd.) at 37 °C for 20 h, followed by RDE inactivation at 56 °C for 30–60 min. The treated sera were serially diluted two-fold with PBS in 96-well U-bottom microtiter plates and mixed with the amount of virus equivalent to eight hemagglutination units, followed by incubation at room temperature (25 °C) for 60 min. After addition of 50 μl of 0.5% chicken red blood cells, the mixtures were gently mixed and incubated at 4 °C for a further 45 min. HI titers are expressed as the inverse of the highest antibody dilution that inhibited hemagglutination.

NA inhibition assay. *In vitro* NA activity of viruses was determined as described previously.[23,24] Briefly, 2′-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid (MUNANA; Sigma, St Louis, MO) at a final concentration of 0.1 mmol/L was used as a fluorescent substrate. Dilutions of viruses containing NA activity equivalent to 800–1200 fluorescence units was used in this study. Ten microliters of the diluted viruses and 10 μL of the neuraminidase inhibitor (0.01 nmol/L to 10 μmol/L) in calcium-MES buffer (33 mmol/L 2-[N-morpholino]ethanesulfonic acid, 4 mmol/L $CaCl_2$, pH 6.0) were mixed and incubated at 37°C for 30 minutes, followed by the addition of 30 μL of the substrate. The mixtures were further

incubated at 37°C for 1 h, and the reaction was stopped by adding 150 μL of 0.1 mol/L sodium hydroxide in 80% ethanol (pH 10.0). The fluorescence of the solution was measured at an excitation wavelength of 360 nm and an emission wavelength of 465 nm. The relationship between the concentration of inhibitor and the percentage of fluorescence inhibition was determined and 50% inhibitory concentration (IC_{50}) values were obtained by extrapolating those findings. The IC_{50} values were calculated according to the IC_{50} analysis protocol provided by the Health Protection Agency, London, UK.[25]

Table 1. List of primers and probes used in this study

Primer or probe	Target gene	Sequence (5' - 3')[†]	Orientation
BHA1-N	Influenza B virus HA	AATATCCACAAAATGAAGGC	Forward
BHA2-1867-1887R	Influenza B virus HA	AGTAGTAACAAGAGCATTTTT	Reverse
BNA-F5v2	Influenza B virus NA	TCAAAACTGAAGCAAATAGGCCA	Forward
BNA-R1498-1472	Influenza B virus NA	AATAGGAACAAAGGGTTTAGAACAGA	Reverse
qPCR-B/Victoria-HA-F	B/Victoria virus HA	CCTGTTACATCTGGGTGCTTTCCTATAATG	Forward
qPCR-B/Victoria-HA-R	B/Victoria virus HA	GTTGATARCCTGATATGTTCGTATCCTCKG	Reverse
qPCR-B/Yamagata-HA-F	B/Yamagata virus HA	CCTGTTACATCCGGGTGCTTYCCTATAATG	Forward
qPCR-B/Yamagata-HA-R	B/Yamagata virus HA	GTTGATAACCTKATMTTTTCATATCCTCTG	Reverse
FAM-B/Victoria-HA-Probe	B/Victoria virus HA	(FAM)TTAGACAGCTGCCTAACC(BHQ-1)	
FAM-Type B HA Victoria	B/Victoria virus HA	(FAM)TTAGACAGCTGCCTAACC(MGB/TAMRA)	
HEX-B/Yamagata-HA-Probe	B/Yamagata virus HA	(HEX)TCAGGCAACTASCCAATC(BHQ-1)	
FAM-Type B HA Yamagata	B/Yamagata virus HA	(FAM)TCAGGCAACTASCCAATC(MGB/TAMRA)	

[†]FAM, 6-carboxyfluorescein; HEX, hexacholoro-6-carboxyfluorescein; BHQ-1, black hole quencher; MGB, minor groove binder; TAMRA, 6-carboxytetramethylrhodamine.

RESULTS

Detection of influenza B viruses in Japan during the 2017–2018 and 2018–2019 seasons. During the 2017–2018 and 2018–2019 seasons, a total of 554 respiratory specimens were collected from patients with an influenza-like illness in Tokyo, Japan, and 108 were confirmed to be influenza B virus-positive by real-time RT-PCR using lineage-specific primers/probes. Of 97 influenza B virus-positive samples collected during the 2017–2018 season, 95 (97.9%) belonged to the B/Yamagata-lineage and two (2.1%) to the B/Victoria-lineage. These results are consistent with the National Institute of Infectious Diseases (NIID) report that influenza B viruses of the B/Yamagata- and B/Victoria-lineages co-circulated in Japan during the winter season with the B/Yamagata-lineage predominating.[26] Of 11 influenza B virus-positive samples collected during the 2018–2019 season, two were of the B/Yamagata-linage and nine were of the B/Victoria-lineage. All 108 influenza B viruses were isolated from the clinical samples by inoculation to MDCK, AX4, or hCK cells. Of the 108 influenza B isolates, 68 (62 from the 2017–2018 season and 6 from the 2018–2019 season) were selected for further characterization (**Tables 2 and 3**).

Genetic characterization of influenza B viruses isolated in Japan during the 2017–2018 and 2018–2019 seasons. Recent B/Yamagata-lineage viruses have been classified into two distinct genetic clades based on the phylogenetic characterization of the HA gene: clade 2 and

clade 3, represented by the vaccine reference strains B/Massachusetts/2/2012 and B/Wisconsin/01/2010, respectively.[27] In contrast, recent B/Victoria-lineage viruses belong to clade 1A, which is represented by the vaccine reference strain B/Brisbane/60/2008. To determine the HA gene clade of the 68 influenza B viruses isolated in Japan during the 2017–2018 and 2018–2019 seasons, I performed phylogenetic analysis of their HA genes together with those of other viruses from the GISAID EpiFlu database. This analysis showed that all 61 B/Yamagata-lineage virus isolates fell into clade 3 along with the vaccine strain B/Phuket/3073/2013, which was used in Japan for the 2017–2018 and 2018–2019 seasons (**Fig. 1**). Of the 61 B/Yamagata-lineage virus isolates, 26 viruses were recovered from patients who were vaccinated for the 2017–2018 or 2018–2019 seasons (**Table 2**). Four major antigenic sites have been identified in the globular head domain of influenza B virus HA: the 120-loop, 150-loop, 160- loop, and 190-helix.[28] Although several amino acid changes were detected in the antigenic sites of HA, none was shared among all of the isolates from the vaccinated patients (**Fig. 1**).

The seven B/Victoria-lineage isolates possessed HA genes that belonged to clade 1A along with the vaccine strains B/Texas/02/2013 (B/Brisbane/60/2008-like virus) and B/Maryland/15/2016 (B/Colorado/06/2017-like virus), which were used in Japan for the 2017–2018 and the 2018–2019 seasons, respectively (**Fig. 2**). B/Colorado/06/2017 contains a two-amino-acid deletion at positions 162 and 163 in its HA. Two isolates of the 2017–2018 season

and two isolates of the 2018–2019 season had this two-amino-acid deletion. The remaining three isolates of the 2018–2019 season harbored a three-amino-acid deletion at positions 162–164 of HA. B/Victoria-lineage viruses with the three-amino-acid deletion fell into two distinct subgroups (**Fig 2**): one subgroup shared an amino acid substitution at position K136E; the other subgroup had two common amino acid substitutions at I180T and K209N. All three viruses with the three-amino-acid deletion that were isolated during the 2018–2019 season in Japan belonged to the former subgroup. Of the seven B/Victoria-lineage isolates, three were isolated from patients who were vaccinated for the 2018–2019 season (**Table 3**). Although several amino acid substitutions were detected in the antigenic sites of HA, none of them were shared among the three isolates from the vaccinated patients (**Fig. 2**).

Table 2. Influenza B viruses isolated during the 2017–2018 season analyzed in this study

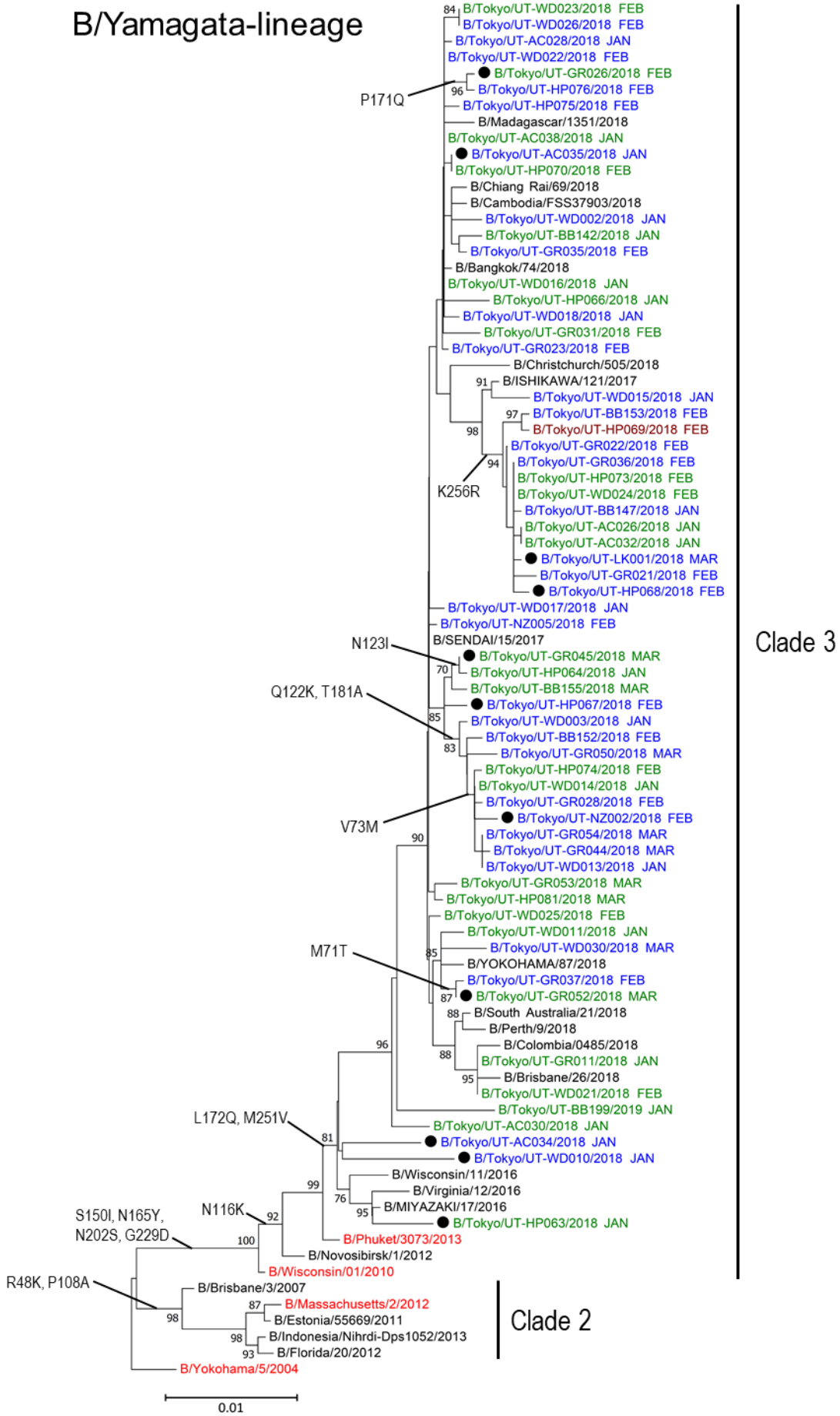
		Patients information[†]		
Strain Name	Lineage	Sex	Age (years)	Influenza vaccination status for the 2017–2018 season
B/Tokyo/UT-AC026/2018	B/Yamagata	Male	42	Vaccinated
B/Tokyo/UT-AC028/2018	B/Yamagata	Male	17	Unvaccinated
B/Tokyo/UT-AC030/2018	B/Yamagata	Female	14	Vaccinated
B/Tokyo/UT-AC032/2018	B/Yamagata	Female	41	Vaccinated
B/Tokyo/UT-AC034/2018	B/Yamagata	Female	50	Unvaccinated
B/Tokyo/UT-AC035/2018	B/Yamagata	Female	17	Unvaccinated
B/Tokyo/UT-AC038/2018	B/Yamagata	Female	5	Vaccinated
B/Tokyo/UT-BB142/2018	B/Yamagata	Male	3	Vaccinated
B/Tokyo/UT-BB147/2018	B/Yamagata	Female	4	Unvaccinated
B/Tokyo/UT-BB152/2018	B/Yamagata	Male	15	Unvaccinated
B/Tokyo/UT-BB153/2018	B/Yamagata	Male	9	Unvaccinated
B/Tokyo/UT-BB155/2018	B/Yamagata	Male	12	Vaccinated
B/Tokyo/UT-GR011/2018	B/Yamagata	Female	14	Vaccinated
B/Tokyo/UT-GR021/2018	B/Yamagata	Female	60	Unvaccinated
B/Tokyo/UT-GR022/2018	B/Yamagata	Female	4	Unvaccinated
B/Tokyo/UT-GR023/2018	B/Yamagata	Female	8	Unvaccinated
B/Tokyo/UT-GR026/2018	B/Yamagata	Female	12	Vaccinated
B/Tokyo/UT-GR028/2018	B/Yamagata	Male	13	Unvaccinated
B/Tokyo/UT-GR031/2018	B/Yamagata	Male	15	Vaccinated
B/Tokyo/UT-GR035/2018	B/Yamagata	Male	63	Unvaccinated
B/Tokyo/UT-GR036/2018	B/Yamagata	Female	14	Unvaccinated
B/Tokyo/UT-GR037/2018	B/Yamagata	Male	21	Unvaccinated
B/Tokyo/UT-GR044/2018	B/Yamagata	Female	16	Unvaccinated
B/Tokyo/UT-GR045/2018	B/Yamagata	Female	27	Vaccinated
B/Tokyo/UT-GR050/2018	B/Yamagata	Male	17	Unvaccinated
B/Tokyo/UT-GR052/2018	B/Yamagata	Male	18	Vaccinated
B/Tokyo/UT-GR053/2018	B/Yamagata	Female	8	Vaccinated
B/Tokyo/UT-GR054/2018	B/Yamagata	Female	34	Unvaccinated
B/Tokyo/UT-HP063/2018	B/Yamagata	Male	42	Vaccinated
B/Tokyo/UT-HP064/2018	B/Yamagata	Female	43	Vaccinated
B/Tokyo/UT-HP066/2018	B/Yamagata	Male	46	Vaccinated
B/Tokyo/UT-HP067/2018	B/Yamagata	Female	75	Unvaccinated
B/Tokyo/UT-HP068/2018	B/Yamagata	Female	29	Unvaccinated
B/Tokyo/UT-HP069/2018	B/Yamagata	N/A	N/A	N/A

B/Tokyo/UT-HP070/2018	B/Yamagata	Male	47	Vaccinated
B/Tokyo/UT-HP073/2018	B/Yamagata	Female	85	Vaccinated
B/Tokyo/UT-HP074/2018	B/Yamagata	Female	70	Vaccinated
B/Tokyo/UT-HP075/2018	B/Yamagata	Male	33	Unvaccinated
B/Tokyo/UT-HP076/2018	B/Yamagata	Female	62	Unvaccinated
B/Tokyo/UT-HP081/2018	B/Yamagata	Female	71	Vaccinated
B/Tokyo/UT-NZ002/2018	B/Yamagata	Male	17	Unvaccinated
B/Tokyo/UT-NZ005/2018	B/Yamagata	Female	87	Unvaccinated
B/Tokyo/UT-LK001/2018	B/Yamagata	Male	6	Unvaccinated
B/Tokyo/UT-WD002/2018	B/Yamagata	Female	8	Unvaccinated
B/Tokyo/UT-WD003/2018	B/Yamagata	Male	12	Unvaccinated
B/Tokyo/UT-WD010/2018	B/Yamagata	Female	28	Unvaccinated
B/Tokyo/UT-WD011/2018	B/Yamagata	Male	16	Vaccinated
B/Tokyo/UT-WD013/2018	B/Yamagata	Female	13	Unvaccinated
B/Tokyo/UT-WD014/2018	B/Yamagata	Female	14	Vaccinated
B/Tokyo/UT-WD015/2018	B/Yamagata	Male	8	Unvaccinated
B/Tokyo/UT-WD016/2018	B/Yamagata	Male	19	Vaccinated
B/Tokyo/UT-WD017/2018	B/Yamagata	Male	14	Unvaccinated
B/Tokyo/UT-WD018/2018	B/Yamagata	Male	51	Unvaccinated
B/Tokyo/UT-WD021/2018	B/Yamagata	Female	11	Vaccinated
B/Tokyo/UT-WD022/2018	B/Yamagata	Female	14	Unvaccinated
B/Tokyo/UT-WD023/2018	B/Yamagata	Female	9	Vaccinated
B/Tokyo/UT-WD024/2018	B/Yamagata	Female	6	Vaccinated
B/Tokyo/UT-WD025/2018	B/Yamagata	Female	N/A	Vaccinated
B/Tokyo/UT-WD026/2018	B/Yamagata	Male	12	Unvaccinated
B/Tokyo/UT-WD030/2018	B/Yamagata	Male	9	Unvaccinated
B/Tokyo/UT-WD028/2018	B/Victoria	Female	49	Unvaccinated
B/Tokyo/UT-WD029/2018	B/Victoria	Male	N/A	Unvaccinated

[†]N/A, not available.

Table 3. Influenza B viruses isolated during the 2018–2019 season analyzed in this study

		Patient information		
Strain Name	Lineage	Sex	Age (year)	Influenza vaccination status for the 2018–2019 season
B/Tokyo/UT-BB199/2019	B/Yamagata	Male	3	Vaccinated
B/Tokyo/UT-BB200/2019	B/Victoria	Female	6	Vaccinated
B/Tokyo/UT-BB207/2019	B/Victoria	Male	1	Vaccinated
B/Tokyo/UT-BB241-0/2019	B/Victoria	Male	7	Vaccinated
B/Tokyo/UT-BB248-0/2019	B/Victoria	Female	10	Unvaccinated
B/Tokyo/UT-WD050-0/2019	B/Victoria	Female	5	Unvaccinated


B/Yamagata-lineage
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B/Tokyo/UT-WD026/2018 FEB
B/Tokyo/UT-AC028/2018 JAN
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B/Tokyo/UT-GR026/2018 FEB
B/Tokyo/UT-HP076/2018 FEB
P171Q
B/Tokyo/UT-HP075/2018 FEB
B/Madagascar/1351/2018
B/Tokyo/UT-AC038/2018 JAN
B/Tokyo/UT-AC035/2018 JAN
B/Tokyo/UT-HP070/2018 FEB
B/Chiang Rai/69/2018
B/Cambodia/FSS37903/2018
B/Tokyo/UT-WD002/2018 JAN
B/Tokyo/UT-BB142/2018 JAN
B/Tokyo/UT-GR035/2018 FEB
B/Bangkok/74/2018
B/Tokyo/UT-WD016/2018 JAN
B/Tokyo/UT-HP066/2018 JAN
B/Tokyo/UT-WD018/2018 JAN
B/Tokyo/UT-GR031/2018 FEB
B/Tokyo/UT-GR023/2018 FEB
B/Christchurch/505/2018
B/ISHIKAWA/121/2017
B/Tokyo/UT-WD015/2018 JAN
B/Tokyo/UT-BB153/2018 FEB
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B/Wisconsin/01/2010
R48K, P108A
B/Brisbane/3/2007
B/Massachusetts/2/2012
B/Estonia/55669/2011
B/Indonesia/Nihrdi-Dps1052/2013
B/Florida/20/2012
B/Yokohama/5/2004
0.01
Clade 3
Clade 2

Fig 1. Phylogenic tree of the HA genes of B/Yamagata-lineage viruses isolated in Japan during the 2017–2018 and 2018–2019 influenza seasons. The tree was built using the neighbor joining method with 1,000 bootstrap replicates. Bootstrap values ≥70 are displayed on the branches. The month during which the clinical specimens were collected is shown after each strain name. The scale bar shows a 1% nucleotide change between close relatives. Influenza B isolates examined in this study are shown in green, blue, and brown. Green and blue indicate viruses recovered from vaccinated and unvaccinated patients in the 2017–2018 or 2018–2019 influenza seasons, respectively. Brown shows viruses isolated from a patient whose influenza vaccination records were not available. Vaccine and vaccine-related strains are indicated in red. The black circle symbol indicated the viruses that were subjected to the HI assay.

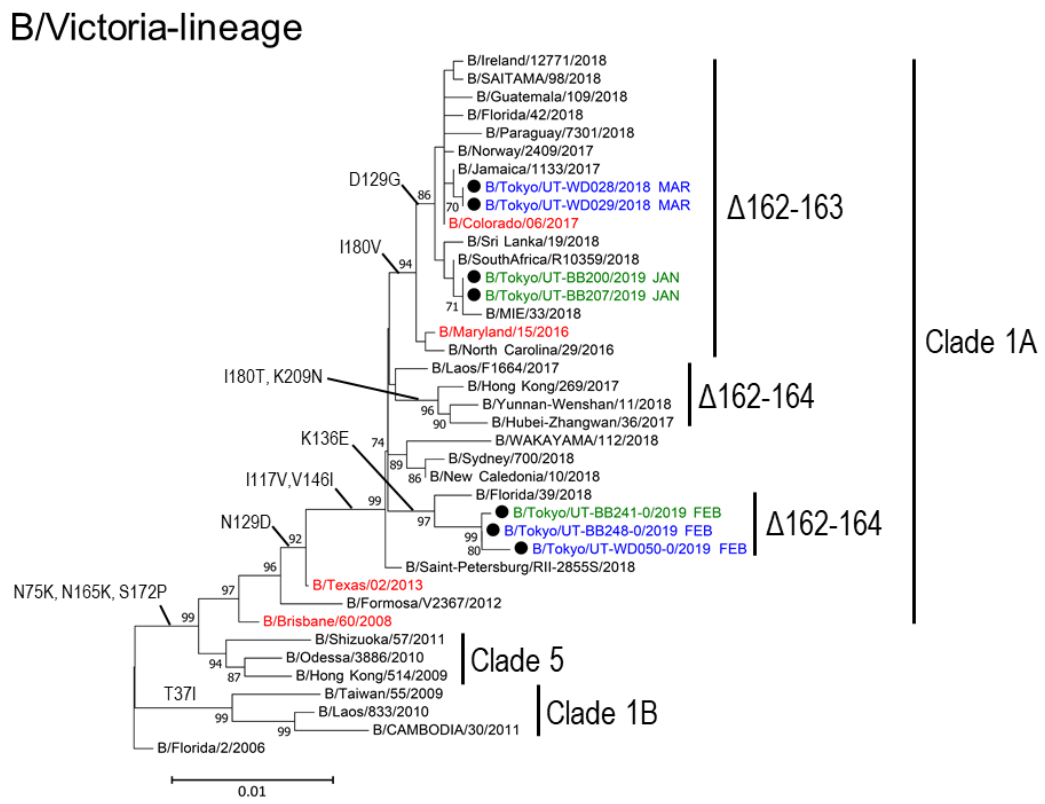

Fig 2. Phylogenic tree of the HA genes of B/Victoria-lineage viruses isolated in Japan during the 2017–2018 and 2018–2019 influenza seasons. The tree was built using the neighbor joining method with 1,000 bootstrap replicates. Bootstrap values ≥70 are displayed on the branches. The month during which the clinical specimens were collected is shown after each strain name. The scale bar shows a 1% nucleotide change between close relatives. Influenza B isolates examined in this study are shown in green and blue. Green and blue indicate viruses recovered from vaccinated and unvaccinated patients in the 2018–2019 influenza season, respectively. Vaccine and vaccine-related strains are indicated in red. The black circle symbol indicated the viruses that were subjected to the HI assay. The Δ162-163 and Δ162-164 indicate viruses possessing the two-amino-acid deletion at positions 162 and 163 in HA and viruses possessing the three-amino-acid deletion at positions 162–164 in HA, respectively.

Antigenic characterization of influenza B viruses isolated in Japan during the 2017–2018 and 2018–2019 seasons. To elucidate whether influenza B viruses isolated in Japan during the 2017–2018 and 2018–2019 influenza seasons were antigenically related to the vaccine strains used for these two seasons in Japan, I performed an HI assay using post-infection ferret antisera raised against cell culture-propagated vaccine or vaccine-related strains.[29] For the B/Yamagata-lineage viruses, all 11 isolates tested, which had amino acid substitutions in the globular head domain of their HA compared with the B/Phuket/3073/2013 virus, were readily recognized by the ferret antisera raised against the cell culture-propagated vaccine viruses B/Wisconsin/01/2010 and B/Phuket/3073/2013 at titers within 2-fold of the homologous titer of these antisera and were less well recognized by the ferret antisera raised against the cell culture-propagated vaccine virus B/Massachusetts/2/2012 (titers within 2- or 4- fold of the homologous titer) (**Table 4**). However, like the clade 3 reference viruses B/Wisconsin/01/2010 and B/Phuket/3073/2013, these 11 isolates were poorly recognized by the ferret antisera raised against cell culture-propagated B/Yokohama/5/2004, which is genetically closely related to the vaccine virus B/Florida/4/2006, with titers 16- or 32- fold lower than the homologous titer. These results indicate that all tested B/Yamagata-lineage isolates were antigenically and genetically closely related to the vaccine strains B/Wisconsin/01/2010 and B/Phuket/3073/2013.

Table 4. Antigenic analysis of B/Yamagata-lineage viruses isolated in Japan during the 2017–2018 season

Virus	Genetic clade	Hemagglutination inhibition titer			
		Post-infection ferret antisera			
		B/Yokohama/5/2004	B/Wisconsin/01/2010	B/Massachusetts/2/2012	B/Phuket/3073/2013
Reference viruses					
B/Yokohama/5/2004	1	**1280**	640	640	320
B/Wisconsin/01/2010	3	80	**640**	320	320
B/Massachusetts/2/2012	2	640	320	**640**	160
B/Phuket/3073/2013	3	80	320	320	**320**
Test viruses					
B/Tokyo/UT-AC034/2018	3	80	320	320	320
B/Tokyo/UT-AC035/2018	3	80	320	160	160
B/Tokyo/UT-GR026/2018	3	80	320	160	160
B/Tokyo/UT-GR045/2018	3	40	320	160	160
B/Tokyo/UT-GR052/2018	3	40	320	160	160
B/Tokyo/UT-HP063/2018	3	80	320	320	320
B/Tokyo/UT-HP067/2018	3	80	320	320	320
B/Tokyo/UT-HP068/2018	3	80	320	320	160
B/Tokyo/UT-NZ002/2018	3	80	640	320	320
B/Tokyo/UT-LK001/2018	3	80	320	320	160
B/Tokyo/UT-WD010/2018	3	80	640	320	320

Homologous titers are underlined and bolded.

For B/Victoria-lineage viruses, all 7 isolates were subjected to an HI assay. All 7 of the tested isolates were poorly recognized by the ferret antiserum raised against the cell culture-propagated vaccine strain B/Texas/02/2013, with titers 8- or 16-fold lower than the homologous titer of this antiserum (**Tables 5 and 6**). An antiserum raised against the cell culture-propagated vaccine strain B/Brisbane/60/2008 recognized the 7 isolates but at 4-, 8-, or 16-fold reduced levels compared with the titers of the antiserum for the homologous virus. In contrast, antisera raised against cell culture-propagated B/Colorado/06/2017 efficiently inhibited the hemagglutination activity of the four test viruses lacking two amino acids in their HAs, with titers similar to that of the homologous virus, consistent with the WHO report.[30] However, the three test viruses carrying the three-amino-acid deletion were recognized less well by the antiserum against B/Colorado/06/2017 with titers 4- or 8-fold lower than the homologous titer of this antiserum. These results indicate that the four test viruses with the two-amino-acid deletion are antigenically and genetically closely related to the vaccine virus B/Colorado/06/2017, but the remaining three test viruses with the three-amino-acid deletion may be antigenically different from the vaccine strain.

Table 5. Antigenic analysis of B/Victoria-lineage viruses isolated in Japan during the 2017–2018 season

Virus	Genetic clade	Hemagglutination inhibition titer		
		Post-infection ferret antisera		
		B/Brisbane/60/2008	B/Texas/02/2013	B/Colorado/06/2017
Reference viruses				
B/Brisbane/60/2008	1A	**2560**	1280	320
B/Texas/02/2013	1A	1280	**1280**	320
B/Colorado/06/2017	1A (Δ2 aa)	640	160	**1280**
Test viruses				
B/Tokyo/UT-WD028/2018	1A (Δ2 aa)	160	80	1280
B/Tokyo/UT-WD029/2018	1A (Δ2 aa)	320	160	1280

Homologous titers are underlined and bolded.

Table 6. Antigenic analysis of B/Victoria-lineage viruses isolated in Japan during the 2018–2019 season

Virus	Genetic clade	Hemagglutination inhibition titer		
		Post-infection ferret antisera		
		B/Brisbane/60/2008	B/Texas/02/2013	B/Colorado/06/2017
Reference viruses				
B/Brisbane/60/2008	1A	**2560**	640	320
B/Texas/02/2013	1A	1280	**1280**	160
B/Colorado/06/2017	1A (Δ2 aa)	640	160	**1280**
Test viruses				
B/Tokyo/UT-BB200/2019	1A (Δ2 aa)	640	80	1280
B/Tokyo/UT-BB207/2019	1A (Δ2 aa)	640	160	1280
B/Tokyo/UT-BB241-0/2019	1A (Δ3 aa)	640	80	320
B/Tokyo/UT-BB248-0/2019	1A (Δ3 aa)	320	80	160
B/Tokyo/UT-WD050-0/2019	1A (Δ3 aa)	320	80	160

Homologous titers are underlined and bolded.

Antiviral susceptibility. To monitor the susceptibility of influenza B viruses to NA inhibitors in Japan during the 2017–2018 and 2018–2019 seasons, the nucleotide sequences of the NA segments of the 68 isolates were determined by means of Sanger sequencing. Sequence analysis revealed no mutations known to confer resistance to NA inhibitors in the influenza B isolates,[15,16,31,32,33,34,35,36,37,38] except for one B/Yamagata-lineage isolate (B/Tokyo/UT-AC032/2018) that possessed a G407S mutation in its NA.[23] A fluorescence NA inhibition assay with the MUNANA substrate was used to characterize the susceptibility of B/Tokyo/UT-AC032/2018 virus to oseltamivir carboxylate, peramivir, zanamivir, and laninamivir (**Table 7**). The isolate showed a marked reduction in susceptibility to peramivir, zanamivir, and laninamivir (64-, 167-, and 204- fold increases in IC_{50} values, respectively, compared with a drug-susceptible control virus, a recombinant virus possessing the HA and NA genes from B/Phuket/3073/2013). This isolate also exhibited moderately reduced susceptibility to oseltamivir carboxylate (8-fold).

Table 7. Virus sensitivity to NA inhibitors in vitro†

Virus	NA change	IC_{50} values (nM) of NA inhibitors (fold differences‡)			
		Oseltamivir carboxylate§	Zanamivir	Peramivir	Laninamivir¶
rPhuket3073/Yamagata††	wild-type	64.9 ± 11.7 (1.0)	5.9 ± 1.1 (1.0)	0.5 ± 0.1 (1.0)	2.5 ± 0.1 (1.0)
rPhuket3073/Yamagata-E117A‡‡	E117A	38,075.9 ± 7,365.0 (586.6)	164,197.3 ± 29,976.2 (27830.0)	14,959.5 ± 296.9 (29,919.0)	54,022.7 ± 4,659.7 (21,609.1)
B/Tokyo/UT-AC032/2018	G407S	537.2 ± 215.7 (8.3)	986.6 ± 507.9 (167.2)	32.1 ± 12.9 (64.2)	510.1 ± 244.4 (204.0)

†IC50 values were determined by using an NA-Fluor Influenza Neuraminidase Assay Kit. Average IC50 values and standard deviations were calculated from three independent assays performed in duplicate.

‡Compared with IC50 values obtained with the sensitive control rPhuket3073/Yamagata.

§Oseltamivir carboxylate is the active form of oseltamivir.

¶Laninamivir is the active form of laninamivir octanoate.

††A recombinant virus possessing the HA and NA genes from B/Phuket/3073/2013 and the remaining genes from B/Yamagata/1/73 virus.

‡‡A recombinant Phuket3073/Yamagata virus with an E117A mutation in its NA.

DISCUSSION

Most of the B/Victoria-lineage viruses that were detected worldwide during the eight influenza seasons that followed the 2009–2010 season were antigenically closely related to the vaccine strain B/Brisbane/60/2008.[39] In this study, during the 2017–2018 and/or 2018–2019 seasons in Japan, I detected B/Victoria-lineage viruses with a deletion of two or three amino acids at positions 162 and 163 or 162–164 in HA, respectively, which are antigenically distinct from the vaccine strain. Antigenic variants possessing the two-amino-acid deletion were isolated in the United States during the 2016–2017 season,[40] whereas variants with the three-amino-acid deletion were isolated in China and Hong Kong during the 2017–2018 season.[41] Subsequently, these antigenic variants have been reported in many countries.[30,42] The WHO recommended the B/Colorado/06/2017-like virus with the two-amino-acid deletion for the 2019–2020 northern hemisphere influenza vaccine.[30] My antigenic analyses with post-infection ferret sera showed that all tested B/Victoria-lineage viruses with the three-amino-acid deletion were inhibited less well by the antiserum raised against B/Colorado/06/2017 than viruses that possess the two-amino-acid deletion, consistent with the WHO report.[30] These findings suggest that contemporary B/Victoria-lineage viruses with the three-amino-acid deletion may be antigenically distinguishable from viruses with the two-amino-acid deletion. Importantly, an increasing proportion of a variant with the three-amino-acid deletion was reported in many countries during the 2018–2019 season.[30] Therefore, continued monitoring for the prevalence

of B/Victoria-lineage viruses with the three-amino-acid deletion is important in terms of the vaccine virus selection for the 2020-2021 northern hemisphere influenza season.

Influenza B virus viruses carrying the G407S mutation in their NA, which confers reduced susceptibility to NA inhibitors, were sporadically detected in Japan during the 2004–2005 season,[23] and in Australia and the United States during the 2015–2016 season.[38] In this study, I also detected an influenza B virus with the NA-G407S mutation during the 2017–2018 season in Japan (**Table 7**). This virus (B/Tokyo/UT-AC032/2018) was isolated from a specimen collected from a patient prior to drug treatment. Although it is unclear whether the patient had contact with an influenza patient who was treated with NA inhibitors, it is possible that this variant may be transmissible from person to person. Further investigations are required to determine whether this NA-G407S mutation affects the transmissibility of influenza B viruses. This virological surveillance study may have been limited by the fact that samples were collected in a limited area in Japan and by the relatively small number of influenza B isolates. Nevertheless, such monitoring of the most recent circulating strains contributes to the early detection of new antigenic variants and drug-resistant viruses.

CONCLUSIONS

In this study, I assessed the antigenic and genetic characteristics of influenza B viruses isolated in Japan during the 2017–2018 and 2018–2019 influenza seasons. All tested B/Yamagata-lineage isolates were antigenically and genetically closely related to B/Phuket/3073/2013, a vaccine strain for these two seasons. All tested B/Victoria-lineage isolates were genetically closely related to B/Texas/02/2013, the recommended vaccine strain for the 2017–2018 season; however, they possessed a deletion of two or three amino acids at positions 162 and 163 or 162–164 in HA, and were antigenically distinct from B/Texas/02/2013. Of these isolates, viruses with the three-amino-acid deletion appeared to be antigenically different from the B/Colorado/06/2017 virus with the two-amino-acid deletion, the recommended vaccine strain for the 2018–2019 season. One B/Yamagata-lineage isolate carrying a G407S mutation in its NA, which confers reduced susceptibility to NA inhibitors, was detected. These results highlight the need for continued monitoring for the prevalence of antigenic variants of influenza B viruses, particularly the variant with the three-amino-acid deletion and the variant with reduced NA inhibitor susceptibility.

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