season and with zanamivir.[9,10] The new NAI, laninamivir, became available from the 2010–2011 season in Japan.[4,12,13] However, the effectiveness of NAIs, including laninamivir, against various types of influenza viruses, including A(H1N1)pdm09, has not been clinically compared in the same season.

In this report, we compare the clinical symptoms of A(H1N1)pdm09 patients in the 2009–2010 and 2010–2011 seasons and also among the A(H1N1)pdm09, A(H3N2), and B influenzas that were circulating in the 2010–2011 season. We analyzed the duration of fever ≥37·5°C after the first dose of oseltamivir or zanamivir in both seasons and for all three NAIs in the 2010–2011 season.[2,6,8,10] The IC_{50} (50% inhibitory concentration) of the three NAIs was determined for the three types of influenza virus in the 2010–2011 season.[7,9,14]

Methods

Study procedures

Family doctors, pediatricians, and physicians at 13 clinics who belong to the Influenza Study Group of the Japan Physicians Association participated in the study. Patients were enrolled from August 11, 2009 through April 6, 2010 (median: November 11, 2009) in the 2009–2010 season and from November 18, 2010 through May 23, 2011 (median: January 31, 2011) in the 2010–2011 season. Patients who reported to any of our 13 clinics with an influenza-like illness manifesting any two of the following symptoms: body temperature ≥37·5°C, rhinorrhea, sore throat, cough, general fatigue, loss of appetite, or headache were tested by commercial antigen detection kit. From all outpatients with influenza, diagnosed by antigen detection kit and without severe underlying diseases such as chronic obstructive pulmonary disease or chronic heart disease, those who received NAIs within 48 h after the onset of symptoms were registered in this study after providing informed consent.

Oseltamivir has been reported to be related to the neuropsychiatric symptoms of young adults and has been prohibited in Japan, in most cases, for use by patients aged from 10 to 19 years, and zanamivir and laninamivir are not recommended for patients with underlying respiratory disease or children under 5 years. Thus, intravenous peramivir was administered to a few patients. The symptoms of these patients were analyzed, but were excluded from the analysis of the duration of fever. The decision of which NAI to administer, oseltamivir, zanamivir, laninamivir, or peramivir, was left to the discretion of the patient's physician, who followed the above guidelines and patient preference.

Specimens from nasal swabs, throat swabs, nasal aspirates, or blown nasal discharge were subjected to antigen detection and virus isolation. Of the commercially available antigen detection kits based on immunochromatography, Imuno Ace Flu [Touns], QuickNavi-Flu [Denka Seiken], and Capilia FluA + B [Alfresa Pharma] were mainly used.

Oseltamivir (75 mg for adults and for children who weighed ≥37·5 kg and 2 mg/kg for children who weighed <37·5 kg) was taken orally twice per day for five days. Zanamivir (10 mg for adults and for children aged five years or over) was inhaled twice per day for five days. Laninamivir (20 mg for children <10 years old and 40 mg for adults or children 10 years and older) was inhaled at one sitting.[13] No antipyretics were administered, but acetaminophen was used temporarily in the case of emergency.

Age, sex, vaccination status, results of the antigen detection test kit, and body temperature were recorded for all patients. The date and time of the onset of fever, the date and time of administration of the NAI, and the resolution of fever were recorded by the physician, patient, or an attending family member. The first time point at which a patient reported a fever (temperature, 37·5°C) was defined as the time of onset. Patients were asked to measure body temperature at least three times per day (8:00 A.M., 2:00 P.M., and 8:00 P.M.). The time at which a body temperature of <37·5°C was attained and maintained for more than 24 hours was defined as the time the patient became afebrile. The highest body temperature during the course of the disease was also recorded. For clinical symptoms other than fever, the presence or absence of the following symptoms were noted by the doctor when influenza was diagnosed, cough, rhinorrhea, myalgia, loss of appetite, and fatigue.

All data were collected using an Internet-based protocol based on a server located in a secure room at the Gifu City Medical Association.[15] The time from the initial administration of an NAI to the resolution of fever (the duration of fever after the first dose of NAI) was calculated automatically in the SQL database.[6,10] All study-related documents and procedures were approved by the institutional review board at Hara-Doi Hospital.

Influenza virus isolation

Clinical samples for viral isolation were obtained from nasal or pharyngeal swab, nasal aspiration, or self-blown nasal discharge. Samples were suspended in a solution for virus preservation (M4-RT medium) and sent to a central laboratory (Mitsubishi Chemical Medience Corporation) where they were kept at 4°C. The collected samples were cultured with Madin-Darby canine kidney (MDCK) cells at 33°C.

Viral types and subtypes

The type and subtype of A(H3N2) or B were determined by RT-PCR using subtype-specific primers as described.[16] In brief, viral RNA was extracted from the viral culture supernatant, and then cDNA was synthesized using reverse transcriptase. PCR was carried out with cDNA using

© 2012 Blackwell Publishing Ltd

primer sets specific for the viral type and subtype. For the A(H1N1)pdm09 virus, the subtype was determined by real-time RT-PCR with a specific primer set and a fluorescent-labeled probe.[17]

Measurement of the IC_{50} of the NA inhibitors

IC_{50} to oseltamivir carboxylate, zanamivir, and laninamivir was determined by a fluorescence-based neuraminidase inhibition assay, as described elsewhere[9,18], with culture supernatants. Laninamivir and zanamivir were provided by Daiichi Sankyo Co., Ltd. Oseltamivir carboxylate was prepared from oseltamivir phosphate extracted from the commercial preparation Tamiflu® (Chugai Pharmaceutical Co., Ltd., Tokyo, Japan).

Statistical analysis

The Student's *t*-test was used for between-group comparisons of the peak body temperature, the duration of fever, age, the time from the onset to the first visit, and IC_{50}. The chi-square test was also performed to compare between-group differences in the percentage of patients. Multiple regression analysis was performed to determine which factors affected the duration of fever, such as age, sex, vaccination status, the peak body temperature, the influenza type or subtype, the drug administered, and the time from the onset to the start of treatment. A P value $<0{\cdot}05$ was considered statistically significant.

Results

Patient characteristics

A total of 442 patients were enrolled in the 2009–2010 season as were 415 in the 2010–2011 season. The complete data of 753 patients with influenza were available for analysis: 365 patients with A(H1N1)pdm09 aged 1 to 78 years old in the 2009–2010 season and 199 patients with A(H1N1)pdm09 aged 1 to 81 years old, 96 patients with A(H3N2) aged 1–74 years old, and 93 patients with B aged 3-66 years old in the 2010–2011 season. The clinical characteristics of the patients are summarized in Table 1.

The mean age was significantly higher for A(H1N1)pdm09 in the 2010–2011 season (25·7 ± 18·4 years) than in the 2009–2010 season (19·0 ± 13·6 years, $P < 0{\cdot}001$) and for A(H3N2) and B in the 2010–2011 season (19·2 ± 19·5 years, $P < 0{\cdot}01$, and 14·9 ± 11·9 years, $P < 0{\cdot}001$, respectively). More female than male patients had influenza B. No significant differences were found in vaccination status or time from the onset to the first visit at a clinic.

Peak body temperature

No significant differences in peak body temperature were found in the age group analysis or for adults over 15 years. However, in children 15 years or younger, the peak body temperature was significantly higher in A(H1N1)pdm09 in the 2010–2011 season (39·3 ± 0·6°C) than in A(H1N1)pdm09 in the 2009–2010 season (39·1 ± 0·7°C, $P < 0{\cdot}05$) and in A(H3N2) and B (39·0 ± 0·7°C, $P < 0{\cdot}01$ and 38·9 ± 0·5°C, $P < 0{\cdot}001$, respectively). (Table 1)

In comparison with the peak body temperature to A(H1N1)pdm09 in both seasons of patient groups 0–9, 10–19, 20–39, and 40 years or over, the temperatures of the 0–9 and 10–19 years' age groups ($P < 0{\cdot}01$ and $P < 0{\cdot}05$, respectively) were significantly higher in the 2010–2011 than in the 2009–2010 season (Figure 1)

Other clinical symptoms

The symptoms at the first visit to the clinic, except for fever, are shown in Table 2. The percentages of patients with cough, rhinorrhea, myalgia, loss of appetite, and fatigue were significantly higher for patients with A(H1N1)pdm09 infection in the 2010–2011 than in the 2009–2010 season. This was also true for A(H3N2), except for loss of appetite. No significant differences in the percentages were found for A(H3N2) and B infection.

Between-season comparison of children (≤15 years) and adults (>15 years) with A(H1N1)pdm09 showed the percentages of all five symptoms to be significantly higher for adults in the 2010–2011 than in the 2009–2010 season (Figure 2). For children, the percentage of patients with loss of appetite or fatigue was significantly higher in the 2010–2011 than in the 2009–2010 season.

Effectiveness of NAIs

The duration of fever after the first dose of oseltamivir, zanamivir, or laninamivir is shown for 365 patients in 2009–2010 and 374 patients in 2010–2011 season. (Table 3) Fourteen patients (5 with A(H1N1)pdm09, 7 with A(H3N2), and 2 with B) to whom peramivir was administered in the 2010–2011 season were excluded from this analysis.

The duration tended to be shorter for A(H1N1)pdm09 in both seasons than for A(H3N2) or B in the 2010–2011 season. No significant differences in the duration were found among oseltamivir, zanamivir, and laninamivir for A(H1N1)pdm09, A(H3N2), and B in the 2010–2011 season. For A(H1N1)pdm09 infection, the duration of fever after starting oseltamivir or zanamivir therapy was slightly, but not significantly, longer in the 2010–2011 season than in the 2009–2010 season.

Multiple regression analysis that included the type of virus and the peak body temperature showed significant relationships with the duration of fever ($P = 0{\cdot}00055$ and 0·00033, respectively). No significance was found for the duration of fever after the first dose of an NAI with the NAI administered, age, sex, vaccination status, or the time from the onset to the start of treatment (Table 4).

© 2012 Blackwell Publishing Ltd

Table 1. Baseline clinical characteristics and peak body temperature of patients 15 years or younger and over 15 years

	2009–2010	2010–2011			*P* value between			
	A(H1N1) pdm09 (a)	A(H1N1) pdm09 (b)	A(H3N2) (c)	B (d)	(a) and (b)	(b) and (c)	(c) and (d)	(b) and (d)
Number of patients	365	199	96	93				
Age, mean years ± SD (range)	19·0 ± 13·6 (1–78)	25·7 ± 18·4 (1–81)	19·2 ± 19·5 (1–74)	14·9 ± 11·9 (3–66)	<0·001	<0·01	NS	<0·001
Male/female	188/177	105/94	58/38	39/54	NS	NS	<0·05	NS
Vaccination* Positive/negative/unknown	74/286/5	45/151/3	31/58/7	27/60/6	NS	NS	NS	NS
Time from the onset To the first visit at clinic (hours)	16·3 ± 11·3	15·4 ± 10·8	15·3 ± 10·8	16·5 ± 11·2	NS	NS	NS	NS
Peak body temperature (°C)	39·0 ± 0·7	39·0 ± 0·7	38·9 ± 0·7	38·9 ± 0·5	NS	NS	NS	NS
≤15 years (*n*)	39·1 ± 0·7 (200)	39·3 ± 0·6 (74)	39·0 ± 0·7 (66)	38·9 ± 0·5 (66)	<0·05	<0·01	NS	<0·001
>15 years (*n*)	38·8 ± 0·6 (165)	38·9 ± 0·7 (125)	38·7 ± 0·7 (30)	38·9 ± 0·5 (27)	NS	NS	NS	NS

*Vaccination for seasonal influenza.
() number of patients.

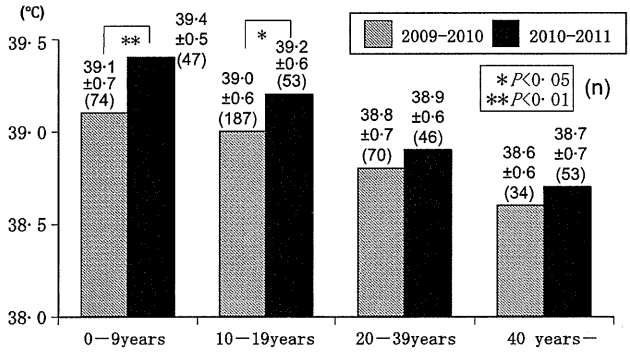

Figure 1. The peak body temperature (°C) of patients with A(H1N1)pdm09 in the 2009–2010 and 2010–2011 seasons, by age. The peak body temperature was significantly higher in the 2010–2011 than the 2009–2010 seasons in the 0–9 and 10–19 years' age groups.

There was no significant difference between the two seasons in the percentage of patients with A(H1N1)pdm09 afebrile at 48 hours after the first dose of oseltamivir or zanamivir (Figure 3).

In the 2010–2011 season, the percentage of patients afebrile at 48 hours after the first dose of laninamivir was significantly higher for A(H1N1)pdm09 (97·1%) than for A(H3N2) and B (81·8%; $P < 0·01$ and 72·2%; $P < 0·001$, respectively) (Figure 3). The percentage after the first dose of oseltamivir was significantly higher for A(H1N1)pdm09 than for B (96·7% and 80·6%, $P < 0·05$). However, no significant difference of duration from the onset to the first dose of an NAI was found between the afebrile and febrile patient groups at 48 hours after the first dose (afebrile and febrile group: 17·1 ± 11·1 and 19·6 ± 14·9 hours in A(H1N1)pdm09, 16·8 ± 11·2 and 18·2 ± 12·1 hours in A(H3N2), and 19·0 ± 10·6 and 16·1 ± 12·5 hours in B, respectively).

In vitro, the IC_{50}s of zanamivir and laninamivir were significantly lower for A(H1N1)pdm09 (0·86 ± 0·32 and 1·77 ± 0·78 nm, respectively) than for A(H3N2) (1·94 ± 0·43 and 3·9 ± 1·6 nm, respectively) or B (12·3 ± 4·0 and 21·3 ± 6·9 nm, respectively). (Table 5) The IC_{50} of oseltamivir was lowest for A(H3N2) (0·74 ± 0·13 nm) and highest for B (44·5 ± 13·6 nm) (Table 5).

Discussion

Cao *et al.* reported that the majority of patients with A(H1N1)pdm09 infection had a mild illness.[19] We also reported that the clinical symptoms of outpatients with A(H1N1)pdm09 infection in the 2009–2010 season tended to be more mild than those of seasonal A(H1N1) in the 2007–2008 and 2008–2009 seasons.[2]

In this study, the peak body temperature was significantly higher in A(H1N1)pdm09 in the 2010–2011 season than in A(H3N2) or B in children 15 years or younger and in A(H1N1)pdm09 in the 2009–2010 season in patients <20 years. The percentage of patients with loss of appetite or fatigue were also higher in the 2010–2011 than in the 2009–2010 season for A(H1N1)pdm09 virus infection in

© 2012 Blackwell Publishing Ltd

Table 2. Percentage of patients with each clinical symptoms at first visit to clinics

	2009–2010	2010–2011			*P* value between			
	A(H1N1) pdm09 (a)	A(H1N1) pdm09 (b)	A(H3N2) (c)	B (d)	(a) and (b)	(b) and (c)	(c) and (d)	(b) and (d)
Number of patients	365	199	96	93				
% of patients with each symptom								
Cough	82·7	90·5	82·3	82·8	<0·05	<0·05	NS	NS
Rhinorrhea	49·6	59·8	81·3	71	<0·05	<0·001	NS	NS
Myalgia	27·4	46·7	18·8	25·8	<0·001	<0·001	NS	<0·001
Loss of appetite	23·3	49·2	56·3	44·1	<0·001	NS	NS	NS
Fatigue	44·1	75·4	61·5	62·4	<0·001	<0·05	NS	<0·05

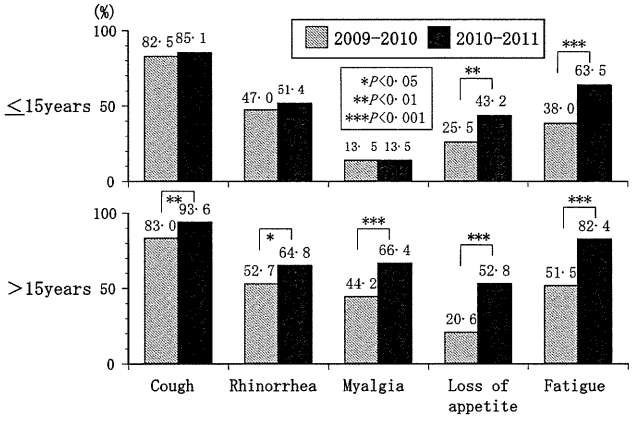

Figure 2. The percentages of the symptoms suffered by patients with A(H1N1) pdm09 infection, by season. The percentage of patients with loss of appetite or fatigue was significantly higher in the 2010–2011 season than in the previous season in children 15 year or younger. The percentage of patients with cough, rhinorrhea, myalgia, loss of appetite, or fatigue was significantly higher in the 2010–2011 season than in the previous season in adults over 15 years.

both the ≤15 years and >15 years' age groups. These results suggest that the severity of symptoms to A(H1N1)pdm09 is increasing as the virus changes from pandemic to seasonal occurrence.

The reason the symptoms to the A(H1N1)pdm09 virus have become slightly more severe is unclear. The percentage of H275Y mutation of A(H1N1)pdm09 in the 2010–2011 season was only 1·1% (2/185) in another of our studies.[4] The virus titer and/or cytokine level may have been increased in this season compared with the previous season. Further study will be necessary. Differences in the season or climate when the A(H1N1)pdm09 was circulating (autumn in the 2009–2010 and winter in the 2010–2011) may also be related to our findings.

We have already reported that oseltamivir was more effective against A(H1N1)pdm09 than against seasonal A(H1N1) in the 2007–2008 and 2008–2009 seasons.[2] We also reported previously that the duration of fever after the first dose of an NAI is significantly correlated, by multiple regression analysis, with the type of virus and peak body

Table 3. The effectiveness of neuraminidaze inhibitors in the 2009–2010 and 2010–2011seasons evaluated by duration of fever

	2009–2010	2010–2011			*P* value between			
Duration of fever after the first dose, hour	**A(H1N1) pdm09 (a)**	**A(H1N1) pdm09 (b)**	**A(H3N2) (c)**	**B (d)**	**(a) and (b)**	**(b) and (c)**	**(c) and (d)**	**(b) and (d)**
Oseltamivir	23·1 ± 12·0 (158)	26·5 ± 10·6 (30)	32·0 ± 19·8 (36)	35·7 ± 25·7 (36)	NS	NS	NS	NS
Zanamivir	26·6 ± 15·0 (207)	29·6 ± 18·2 (59)	33·0 ± 22·1 (22)	30·9 ± 16·8 (19)	NS	NS	NS	NS
Laninamivir	n.a	25·0 ± 15·0 (103)	30·9 ± 21·1 (33)	38·5 ± 26·3 (36)		NS	NS	<0·01

() number of patients.
Fourteen patients [5 with A(H1N1)pdm09, 7 with A(H3N2), and 2 with B] to whom peramivir was administered in the 2010–2011 season were excluded from this analysis.

© 2012 Blackwell Publishing Ltd

Table 4. Results of multiple regression analysis to determine which factors influenced the duration of fever after the first dose

Factor	*P* value
Age	NS
Sex	NS
Vaccination status	NS
Peak body temperature	0·00033
Influenza type or subtype	0·00055
Drug administered	NS
Time from the onset to the start of treatment	NS

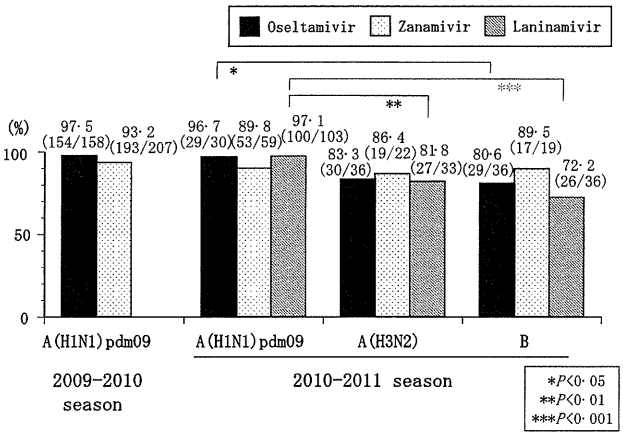

Figure 3. The percentage of patients afebrile at 48 hours after the first dose of each neuraminidase inhibitor. The percentage of patients afebrile at 48 hours after the first dose was significantly higher for A(H1N1)pdm09 than for A(H3N2) (laninamivir) or B (oseltamivir and laninamivir). No significant between-season difference in A(H1N1)pdm09 was found.

temperature, but that there is no correlation with age or the kind of anti-influenza drug.[5] In addition, the effectiveness of vaccination on the duration of fever, as reported in our previous studies, was not confirmed in this study.[5,20]

In this study, the duration of fever and the percentage of patients afebrile at 48 hours after the first dose of oseltamivir or zanamivir did not change significantly from the previous season. However, the duration of fever was significantly shorter for A(H1N1)pdm09 than for B in patients treated with laninamivir, and the percentage of patients afebrile at 48 hours was significantly higher for A(H1N1)pdm09 than for A(H3N2) (laninamivir) or B (oseltamivir and laninamivir).

In our previous study of the 2006–2007 season, the percentages of patients afebrile at 48 hours were 83·1% and 86·7% against influenza A and 55·6% and 80·2% against influenza B for oseltamivir and zanamivir therapy, respectively.[8] In the 2006–2007 season, A(H3N2) was responsible for 90·5% (95/105) of the influenza A cases.[8] The percentage of patients with influenza A(H3N2) afebrile (83·3% and 86·4%, for oseltamivir and zanamivir, respectively) in this study were similar to the data from the 2006–2007 season.

The duration of fever after the first dose of a drug was analyzed to evaluate the clinical effectiveness of these NAIs because it is difficult to evaluate the clinical effectiveness of drugs in outpatient clinics by estimating the mortality rate or incidence of hospitalization. There is a limit to the findings of our study in that it was performed in a general practice setting and not in the context of a rigorous clinical protocol. The body temperature of our outpatients was obtained from reports self-recorded by the patient or a family member. In our previous analysis using this method or virus shedding, oseltamivir was less effective for influenza B than for influenza A and was less effective for A(H1N1) with than without H275Y mutation, especially in children but not so in adults.[9,10] Also, in this study, the duration of fever after oseltamivir therapy tended to be longer in influenza B than in A(H1N1)pdm09 or A(H3N2). However, the difference in the duration between influenza A and B was smaller than in our previous study. The effectiveness of oseltamivir for influenza B compared with A may differ with season. Further study, especially for influenza B, will be necessary.

In this study, we did not compare NAI and non-NAI therapy groups. In Japan, it is unusual to not use an NAI

Table 5. Pre-treatment IC_{50} values for each neuraminidase inhibitor used in the 2010–2011 season

IC_{50} before starting therapy, nm	A(H1N1) pdm09 (a)	A(H3N2) (b)	B (c)	*P* value between (a) and (b)	(b) and (c)	(a) and (c)
Oseltamivir	0·97 ± 0·48 (31)	0·74 ± 0·13 (9)	44·5 ± 13·6 (11)	<0·05	<0·001	<0·001
Zanamivir	0·86 ± 0·32 (31)	1·94 ± 0·43 (9)	12·3 ± 4·0 (11)	<0·001	<0·001	<0·001
Laninamivir	1·77 ± 0·78 (31)	3·9 ± 1·6 (9)	21·3 ± 6·9 (11)	<0·001	<0·001	<0·001

*Duration of fever after the first dose () number of patients.

© 2012 Blackwell Publishing Ltd

for patients with influenza diagnosed by commercial antigen detection kit. The usefulness of NAIs is wide, and NAI therapy is supported by the public medical insurance system. We previously reported that the duration of fever was shorter in NAI therapy than in non-NAI therapy in patients with seasonal influenza.[6,12] We have also reported that the usefulness of oseltamivir and zanamivir for A(H1N1)pdm09 is equal to or higher than for seasonal A(H1N1) without H275Y NA mutation.[2]

The severity of the first and second influenza A(H1N1)pdm09 waves was compared in England.[21–23] Keramarou *et al.*[21] reported more hospital admissions (n = 379) and deaths (n = 26) in Wales in the second wave (peaked in late October, 2009) than in the first wave (n = 44 and only one, respectively; peaked in late July, 2009). Higher mortality rates in the second (September–February) than in the first (June–August) wave were also reported by Presanis *et al.*, (0·025% and 0·015% of patients with A(H1N1)pdm09, respectively) and Mytton *et al.* (5·5 and 1·6 deaths per million population, respectively).[22,23] Our results may coincide with these results; however, accurate comparison is difficult because NAIs are more commonly used in Japan than in England. To our knowledge, no comparison of the severity of A(H1N1)pdm09 virus infection in the first or second waves of the 2009–2010 season and the 2010–2011 season has been reported.

Laninamivir octanoate is inhaled, then converted to laninamivir in the lung, and the binding of laninamivir to virus NA is relatively more stable and lasts longer than has been observed for other NAIs.[13,24] In this study, laninamivir was almost equally as effective as oseltamivir or zanamivir, estimated clinically by the duration of fever; nevertheless, the IC_{50} of laninamivir tended to be higher than that of the other NAIs. Kubo, *et al.* recently reported that 6 days after intranasal administration of 236 μg/kg laninamivir octanoate, the concentration of laninamivir in the lungs of mice was maintained about 730-fold the IC_{50} for A(H1N1)pdm09, 77-fold that of A(H3N2), and 70-fold that of B.[22] In another of our studies, the persistence rates of virus culture 4–6 days after the start of laninamivir therapy were 2·3% (2/86) for A(H1N1)pdm09, 10·5% (2/19) for A(H3N2), and 29·4% (5/17) for B in the 2010–2011 season (Unpublished data by Kawai N, Ikematsu H and Kashiwagi S). Thus, laninamivir has been shown to be more effective against A(H1N1)pdm09 than against either A(H3N2) or B in both *in vitro* and *in vivo* studies. In addition, laninamivir is very convenient to use in outpatient clinics because it can be administered in a single sitting.

In conclusion, although the fever of patients with A(H1N1) pdm09 infection improved quickly with NAI therapy in the 2010–2011 season, the clinical symptoms were more severe than in the 2009–2010 season and more severe than for A(H3N2) or B virus infection. It is notable that the effectiveness of oseltamivir and zanamivir for A(H1N1)pdm09 virus infection has not changed since emergence in 2009 and that the effectiveness of laninamivir for A(H1N1)pdm09 was also high. These NAIs should continue to be recommended, especially for A(H1N1)pdm09 virus infection.

Acknowledgements

We thank Drs Osame Tanaka, Shinro Matsuura, Kenichi Kawamura, Satoshi Yamauchi, Ken-ichi Doniwa, and Kunio Kondou for their support in this study.

References

1 Infectious disease Surveillance Center. Weekly reports of influenza virus isolation/detection. Available at https://hasseidoko.mhlw.go.jp/Byogentai/Pdf/data2e.pdf.

2 Kawai N, Ikematsu H, Tanaka O *et al.* Comparison of the clinical symptoms and the effectiveness of neuraminidase inhibitors for patients with pandemic influenza H1N1 2009 or seasonal H1N1 influenza in the 2007–2008 and 2008–2009 seasons. J Infect Chemother 2011; 17:375–381.

3 WHO. Summary review of the 2010–2011 northern hemisphere winter influenza season. Distribution of virus subtypes by influenza transmission zone (October 2010-April 2011). Available at http://www.who.int/influenza/2010_2011_seasonal_review_map_main.jpg.

4 Ikematsu H, Kawai N, Kashiwagi S. In vitro neuraminidase inhibitory activities of four neuraminidase inhibitors against influenza viruses isolated in the 2010-2011 season in Japan. J Infect Chemother. 2012 Feb 28.

5 Kawai N, Ikematsu H, Iwaki N *et al.* Factors influencing the effectiveness of oseltamivir and amantadine for the treatment of influenza: a multicenter study from Japan of the 2002–2003 influenza season. Clin Infect Dis 2005; 40:1309–1316.

6 Kawai N, Ikematsu H, Iwaki N *et al.* A Comparison of the effectiveness of oseltamivir for the treatment of influenza A and influenza B: a Japanese multicenter study of the 2003–2004 and 2004–2005 influenza seasons. Clin Infect Dis 2006; 43:439–444.

7 Kawai N, Ikematsu H, Iwaki N *et al.* Longer virus shedding in influenza B than in influenza A among outpatients treated with oseltamivir. J Infect 2007; 55:267–272.

8 Kawai N, Ikematsu H, Iwaki N *et al.* A comparison of the effectiveness of zanamivir and oseltamivir for the treatment of influenza A and B. J Infect 2008; 56:51–57.

9 Kawai N, Ikematsu H, Iwaki N *et al.* Clinical effectiveness of oseltamivir for influenza A(H1N1) virus with H274Y neuraminidase mutation. J Infect 2009; 59:207–212.

10 Kawai N, Ikematsu H, Hirotsu N *et al.* Clinical effectiveness of oseltamivir and zanamivir for treatment of influenza A virus subtype H1N1 with the H274Y mutation. A Japanese multicenter study of the 2007–2008 and 2008–2009 Influenza Seasons. Clin Infect Dis 2009; 49:1825–1835.

11 Kawai N, Ikematsu H, Iwaki N *et al.* Persistence of pandemic influenza H1N1 virus in young patients after oseltamivir therapy in the 2009-2010 season: a comparison with seasonal H1N1 with or without H275Y mutation. J Infect Chemother 2012; 18:180–186, DOI 10.1007/s10156-011-0314-2.

12 Watanabe A, Chang SC, Kim MJ, Chu DW, Ohashi Y, MARVEL Study Group. Long-acting neuraminidase inhibitor laninamivir

© 2012 Blackwell Publishing Ltd

octanoate versus oseltamivir for treatment of influenza: A double-blind, randomized, noninferiority clinical trial. Clin Infect Dis 2010; 51:1167–1175.

13 Ikematsu H, Kawai N. Laninamivir octanoate: a new long-acting neuraminidase inhibitor for the treatment of influenza. Expert Rev Anti Infect Ther 2011; 9:851–857.

14 Boivin G, Goyette N. Susceptibility of recent Canadian influenza A and B virus isolates to different neuraminidase inhibitors. Antiviral Res 2002; 54:143–147.

15 Kawai N, Ikematsu H, Iwaki N *et al.* A prospective, Internet-based study of the effectiveness and safety of influenza vaccination in the 2001–2002 influenza season. Vaccine 2003; 21:4507–4513.

16 Stockton J, Ellis JS, Saville M, Clewley JP, Zambon MC. Multiplex PCR for typing and subtyping influenza and respiratory syncytial viruses. J Clin Microbiol 1998; 36:2990–2995.

17 CDC protocol of realtime RT-PCR for influenza A (H1N1). Available at http://www.who.int/csr/resources/publications/swineflu/realtime-ptpcr/en/index.html.

18 Yamashita M, Tomozawa T, Kakuta M, Tokumitsu A, Nasu H, Kubo S. CS-8958, a prodrug of the new neuraminidase inhibitor R-125489, shows long-acting anti-influenza virus activity. Antimicrob Agents Chemother 2008; 53:186–192.

19 Cao B, Li XW, Mao Y *et al.* Clinical features of the initial cases of 2009 pandemic influenza A (H1N1) virus infection in China. N Engl J Med 2009; 361:2507–2517.

20 Ikematsu H, Takeuchi Y, Rosenlund M *et al.* The post-infection outcomes of influenza and acute respiratory infection in patients above 50 years of age in Japan: an observational study. Influenza Other Respi Viruses 2012; 6:211–217.

21 Keramarou M, Cottrell S, Evans MR *et al.* Two waves of pandemic influenza A(H1N1) 2009 in Wales–the possible impact of media coverage on consultation rates, April-December 2009. Euro Surveill 2011; 16:pii: 19772.

22 Presanis AM, Pebody RG, Paterson BJ *et al.* Changes in severity of 2009 pandemic A/H1N1 influenza in England: a Bayesian evidence synthesis. BMJ 2011; 343:d5408, doi: 10.1136/bmj.d5408.

23 Mytton OT, Rutter PD, Mak M, Stanton EA, Sachedina N, Donaldson LJ. Mortality due to pandemic (H1N1) 2009 influenza in England: a comparison of the first and second waves. Epidemiol Infect 2012; 140:1533–41.

24 Kubo S, Tokumitsu A, Tomozawa T, Kakuta M, Yamashita M. High and continuous exposure of laninamivir, an anti-influenza drug, may work suppressively to generate low-susceptibility mutants in animals. J Infect Chemother 2012; 18:69–74.

© 2012 Blackwell Publishing Ltd

J Infect Chemother (2012) 18:529–533
DOI 10.1007/s10156-012-0377-8

ORIGINAL ARTICLE

In vitro neuraminidase inhibitory activities of four neuraminidase inhibitors against influenza viruses isolated in the 2010–2011 season in Japan

Hideyuki Ikematsu · Naoki Kawai · Seizaburo Kashiwagi

Received: 7 November 2011 / Accepted: 23 January 2012 / Published online: 28 February 2012
© Japanese Society of Chemotherapy and The Japanese Association for Infectious Diseases 2012

Abstract The half maximal inhibitory concentration (IC_{50}) of four neuraminidase inhibitors (NAIs), oseltamivir, zanamivir, laninamivir, and peramivir; was measured using influenza viruses isolated in the 2010–2011 influenza season in Japan. Clinical samples for viral isolation were obtained from nasal aspiration, nasopharyngeal swab, or self-blown nasal discharge and cultured with Madin–Darby canine kidney cells. The type and subtype of H3N2 or B were determined by reverse transcriptase polymerase chain reaction (RT-PCR). For the A(H1N1)pdm09 virus, the subtype was determined by real-time RT-PCR. IC_{50}s to oseltamivir carboxylate, zanamivir, laninamivir, and peramivir were determined by a fluorescence-based neuraminidase inhibition assay. Influenza viruses were isolated from 269 patients. A(H1N1)pdm09, H3N2, and B were isolated from 185, 54, and 30 patients, respectively. The geometric means of IC_{50} for oseltamivir were 0.86 and 0.73 nM to A (H1N1) pdm09, except for the two outlier viruses described below and H3N2, respectively, and 33.12 nM for B. The geometric means of IC_{50} for the other three NAIs were lowest to A(H1N1)pdm09 and highest to B. Two A(H1N1)pdm09 isolates showed very high IC_{50} values for oseltamivir (840 and 600 nM) and peramivir (19 and 24 nM). No isolate showed significantly high IC_{50} values for zanamivir or laninamivir. Continuous surveillance against the emergence or spread of influenza virus with high IC_{50} values for anti-influenza drugs is important.

H. Ikematsu (✉)
Department of Clinical Trials, Center for Advanced Medical Innovation, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan
e-mail: hikematsu@camiku.kyushu-u.ac.jp

H. Ikematsu · N. Kawai · S. Kashiwagi
Japan Physicians Association, Tokyo, Japan

Keywords Influenza · Half maximal inhibitory concentration (IC_{50}) · Oseltamivir · Zanamivir · Laninamivir · Peramivir

Introduction

Treating influenza with neuraminidase inhibitors (NAIs) has become the most popular treatment among primary care doctors in Japan. A swine-origin H1N1 strain, A(H1N1) pdm09, was the cause of a pandemic in 2009 [1]. Fortunately, the number of reported influenza-associated deaths was only about 200 in Japan, far fewer than in other countries [1]. The early start of treatment with NAIs, within 48 h of the onset of the influenza symptoms, may have contributed to mitigating symptoms and preventing severe disease.Two NAIs, oseltamivir (Chugai Pharmaceutical Co., Ltd., Tokyo, Japan) and zanamivir (GlaxoSmithKline K.K. Tokyo, Japan), are commonly used in Japanese clinics. The clinical effectiveness of anti-influenza drugs has been confirmed in clinical settings [2–4]. Recently, two new NAIs, laninamivir (Daiichi Sankyo Co., Ltd., Tokyo, Japan) and peramivir (Shionogi & Co., Ltd., Osaka, Japan), were added to the options for influenza treatment in Japan. However, as these various NAIs have been available in the market, drug resistance has become of important clinical concern. An A/H1N1 oseltamivir-resistant strain with a mutation at position 275 of NA was reported in Europe in 2007, and it quickly spread throughout the world [5]. Almost all seasonal A/H1N1 viruses have acquired resistance to oseltamivir worldwide [6]. It has been reported that the H275Y mutant reduces sensitivity to oseltamivir by several hundred-fold in vitro [7]. Reduced clinical effectiveness of oseltamivir to H275Y mutated H1N1 viruses compared to the wild-type H1N1 seasonal influenza virus has been confirmed in the clinical

setting [8, 9]. In addition, the emergence of H275Y mutated A(H1N1)pdm09 with resistance to oseltamivir has been reported [10]. To study the extent of drug resistance, we surveyed the half maximal inhibitory concentration (IC_{50}) of four NAIs, oseltamivir, zanamivir, laninamivir, and peramivir, from influenza viruses isolated in the 2010–2011 influenza season in Japan. The results, including two A(H1N1)pdm09 isolates with significantly high IC_{50} values for oseltamivir and peramivir, but not for zanamivir and laninamivir, are reported.

Materials and methods

Patients

A total of 22 clinics and hospitals from 13 prefectures in Japan participated in this study. Patients were enrolled from 1 November 2010 to 30 April 2011. Samples for viral isolation were collected from patients who showed a positive result by rapid influenza antigen detection kits, based on immunochromatography, with informed consent.

Influenza virus isolation

Clinical samples for viral isolation were obtained from nasal aspiration, nasopharyngeal swab, or self-blown nasal discharge. Samples were suspended with a solution for virus preservation (M4-RT medium, Remel, KS, USA) and sent to a central laboratory (Mitsubishi Chemical Medience Corporation) where they were kept at −80°C. The collected samples were cultured with Madin–Darby canine kidney (MDCK) cells at 33°C.

Viral types and subtypes

The type and subtype of H3N2 or B was determined by amplified DNA size of reverse transcriptase polymerase chain reaction (RT-PCR) using type- and subtype-specific primers as described [11]. In brief, viral RNA was extracted from the clinical sample, then complementary DNA (cDNA) was synthesized using reverse transcriptase. PCR was done with cDNA using primer sets specific for viral type and subtype. For the A(H1N1)pdm09 virus, the subtype was determined by real-time RT-PCR with a specific primer set and a fluorescent-labeled probe (http://www.who.int/csr/resources/publications/swineflu/realtimeptpcr/en/index.html).

Measurement of IC_{50} of NA inhibitors

IC_{50}s to oseltamivir carboxylate, zanamivir, laninamivir, and peramivir were determined by a fluorescence-based neuraminidase inhibition assay with culture supernatants, as described elsewhere [12]. Laninamivir and zanamivir were provided by Daiichi Sankyo Co., Ltd. (Tokyo, Japan). Oseltamivir carboxylate was prepared from oseltamivir phosphate extracted from the commercial preparation Tamiflu® (Chugai Pharmaceutical Co., Ltd., Tokyo, Japan). Peramivir was obtained from the commercially available product (Rapiacta®, Shionogi & Co., Ltd., Osaka, Japan).

Statistical analysis

Difference in age distribution among A(H1N1)pdm09, H3N2, and B patient groups was tested by analysis of variance (ANOVA). Quantitative data were tabulated to provide descriptive summary statistics. Geometric means and 95% confidence intervals (CI) were calculated for IC_{50} values. Box and whisker plots were drawn with log-transformed IC_{50} values by influenza type and subtype. For A(H1N1)pdm09, scatter plots of log-transformed IC_{50} values were made to compare the IC_{50} values of each NAI. P value <0.05 was considered statistically significant. All analyses were performed by SAS® System Release 8.2 (SAS Institute, Cary, NC, USA).

Results

A total of 289 influenza-kit-positive patients were enrolled. Among them, 269 influenza viruses were isolated. Influenza virus A(H1N1)pdm09, H3N2, and B were isolated from 185, 54, and 30 patients, respectively. Age distribution of the patients by virus type and subtype is listed in Table 1. The mean age of the 269 patients who had a virus isolated was 28.1 ± 17.1 years. There was no significant difference in mean ages between males and females. The mean age of A(H1N1)pdm09-positive patients was 30.0 ± 16.2 years, higher than that of H3N2 and B (23.1 ± 18.4 and 21.2 ± 16.5 years, respectively). The difference of age distribution between patients with A(H1N1)pdm09 and H3N2 or B infection was statistically significant ($P = 0.0009$).

The geometric mean of IC_{50} for the four NAIs is listed in Table 2. The geometric mean of IC_{50} for oseltamivir was 0.86 and 0.73 nM to A(H1N1)pdm09, except for the two outlier viruses described below and H3N2, respectively; and 33.12 nM for B. The geometric mean of IC_{50} for the other three NAIs was lowest to A(H1N1)pdm09 and highest to B. The ratio of IC_{50} for B to that of H3N2 for oseltamivir was 45.4 and for zanamivir, laninamivir, and peramivir were 6.8, 6.6, and 6.0, respectively.

The distribution of IC_{50} of the four NAIs is depicted in Fig. 1. The $\log_{10}$ (IC_{50})s of each NAI were distributed in a

Table 1 Distribution of patients by age, sex and virus type

Age group	No. of patients	Males	Females	A(H1N1) pdm09	H3N2	B
0–9	33	14	19	14	13	6
10–19	65	41	24	36	17	12
20–29	54	30	24	43	5	6
30–39	43	24	19	34	6	3
40–49	38	22	16	30	7	1
50–59	25	12	13	22	3	0
60–69	8	2	6	4	3	1
70–79	3	2	1	2	0	1
80+	0	0	0	0	0	0
Total	269	147	122	185	54	30
Mean age ± SD (years)	28.1 ± 17.1	27.5 ± 16.4	28.8 ± 18.0	30.0 ± 16.2	23.1 ± 18.4	21.2 ± 16.5

Data are shown as the number of mean ± standard deviation

Table 2 Half maximal inhibitory concentration (IC_{50}) values of four neuraminidase inhibitors (NAIs) for viral isolates from the 2010–2011 influenza season in Japan

Drug	Geometric mean IC_{50} (nM)		
	A(H1N1)pdm09 (n = 185) Geometric mean (95% CI)	H3N2 (n = 54) Geometric mean (95% CI)	Influenza B (n = 30) Geometric mean (95% CI)
Oseltamivir	0.86 (0.76–0.98)	0.73 (0.65–0.82)	33.12 (28.78–38.09)
Zanamivir	0.73 (0.69–0.78)	1.64 (1.51–1.79)	11.21 (9.98–12.61)
Laninamivir	1.37 (1.27–1.47)	3.22 (2.91–3.56)	21.25 (19.12–23.64)
Peramivir	0.38 (0.34–0.42)	0.66 (0.61–0.71)	3.96 (3.44–4.55)

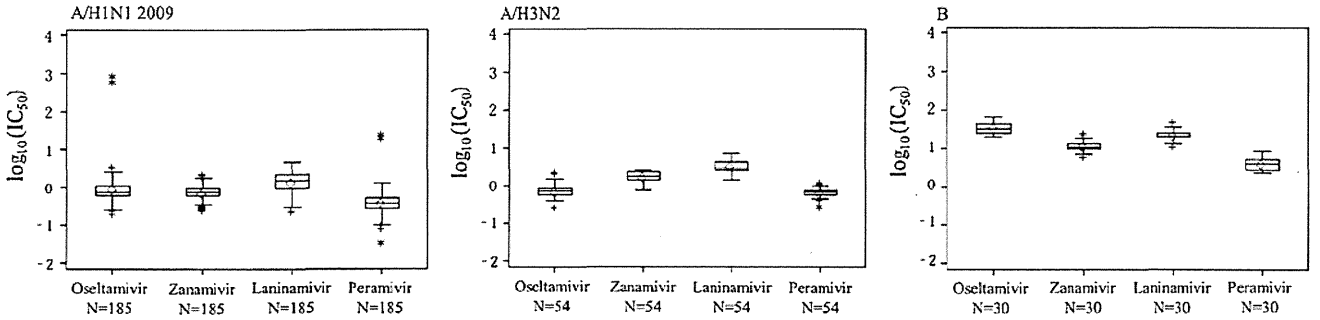

Fig. 1 Half maximal inhibitory concentration (IC_{50}) quartiles of each neuraminidase inhibitor (NAI) for different influenza types. *Diamond* arithmetic mean, *plus symbol* values between 1.5 × IQR and 3 × IQR from UQ/LQ; *asterisk* values above/below 3 × IQR from UQ/LQ, respectively. *IQR* interquartile range, *UQ* 75 percentile, *LQ* 25 percentile

narrow range, except for two viral isolates of A(H1N1)pdm09. The two A(H1N1)pdm09 isolates showed very high IC_{50} values for oseltamivir (840 and 600 nM) and peramivir (19 and 24 nM).

Scatter plots of the log-transformed IC_{50} values of each NAI are shown in Fig. 2. Two isolates showed very high IC_{50} values for oseltamivir but not for zanamivir (Fig. 2a) or laninamivir (Fig. 2b). Two isolates showed high IC_{50} values for both oseltamivir and peramivir (Fig. 2c). No isolate showed a very high IC_{50} value for zanamivir or laninamivir (Fig. 2d). Two isolates showed very high IC_{50} values for peramivir but not for zanamivir (Fig. 2e) or laninamivir (Fig. 2f).

Discussion

In the 2010–2011 season, three influenza strains, A(H1N1) pdm09, H3N2, and B were epidemic in Japan. In this study, A(H1N1)pdm09 was responsible for 68.8% of the isolated viruses. In the 2009–2010 season, almost all clinical isolates were reported to be A(H1N1)pdm09, and patients were mainly 19 years of age and younger. In this study, almost 30% of the patients with A(H1N1)pdm09 were in this age group. The reason for change in the rate of A(H1N1)pdm09 patients in this age group is unknown. For the four NAIs, there was a tendency for the IC_{50} of influenza B virus to be higher than that of A(H1N1)pdm09 and H3N2. The ratio of

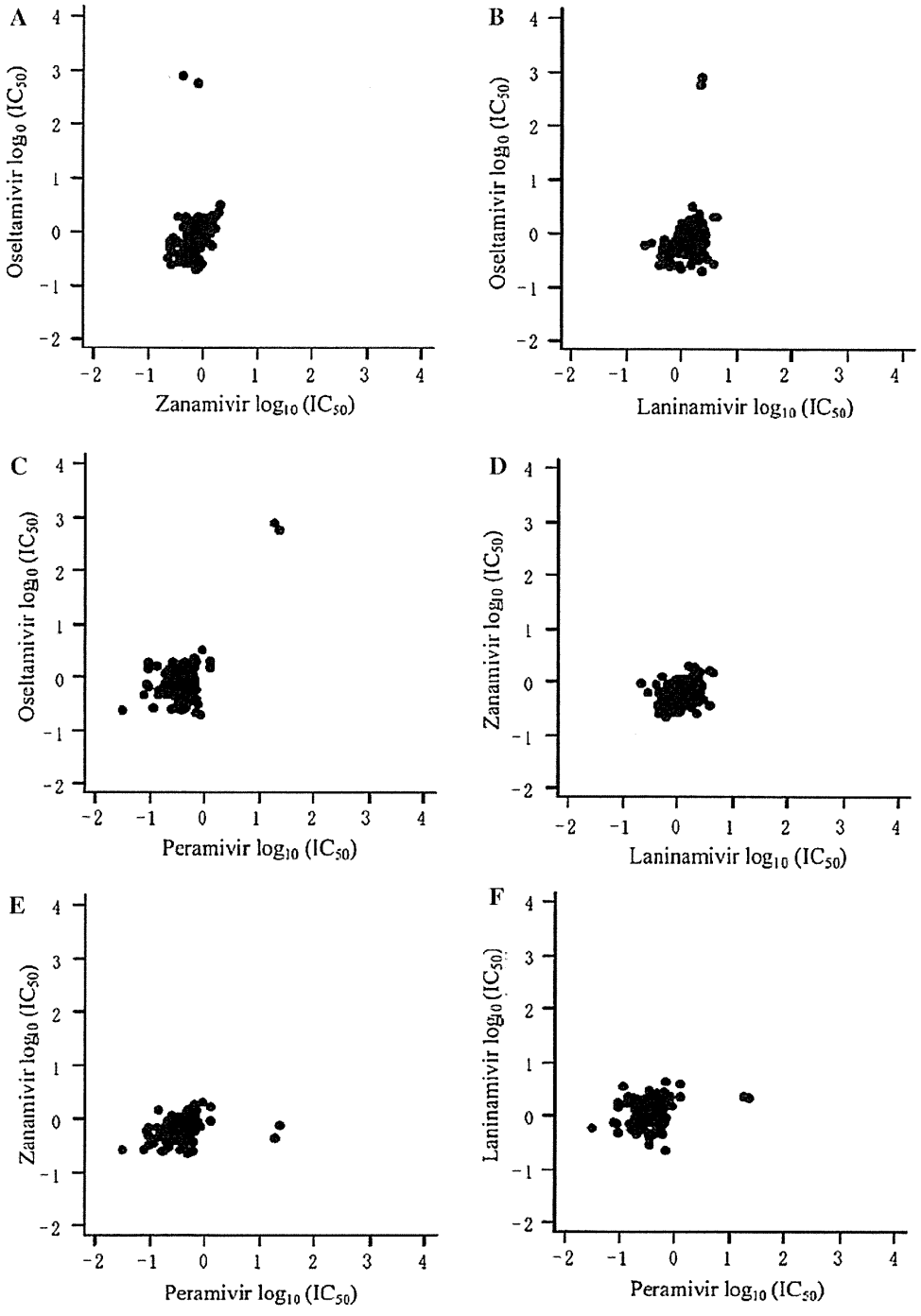

Fig. 2 Scatter plots of Half maximal inhibitory concentration (IC_{50}) values of the four neuraminidase inhibitors (NAIs) for A(H1N1)pdm09

IC_{50} for B to that of H3N2 was especially high in oseltamivir compared with the other three NAIs. It has been reported that the clinical effectiveness of oseltamivir is inferior to influenza B in comparison with influenza A [2]. The clinical efficacy of each drug has not been evaluated in this study. It is plausible that the IC_{50} value or ratio of IC_{50} to viral type and subtype may be useful for predicting the clinical effectiveness of each NAI to a certain viral type or subtype. Further study is necessary to ascertain a relationship between clinical efficacy and IC_{50} value.

The prevalence of oseltamivir-resistant virus was reported to be 1.0% in the 2009–2010 influenza season (http://idsc.nih.go.jp/iasr/graph/tamiful09-10.gif). In this study, two A(H1N1)pdm09 isolates displayed high IC_{50} values for oseltamivir, and the prevalence of oseltamivir resistant virus was calculated at 0.74% of all isolates and 1.1% of A(H1N1)pdm09 isolates. No significant increase in oseltamivir-resistant A(H1N1)pdm09 was observed. However, the existence of oseltamivir-resistant viruses is important; thus, continuous surveillance is necessary. Two

A(H1N1)pdm09 isolates displayed high IC_{50} values for oseltamivir and peramivir, but not for zanamivir and laninamivir. The emergence of A(H1N1)pdm09 viruses with high IC_{50} values has been reported for pediatric patients treated with oseltamivir (http://idsc.nih.go.jp/iasr/rapid/pr3641.html, in Japanese). The molecular basis for H275Y resistance to N1 was described in a structural study of the mutant enzyme [13]. Conformational change induced by the H275Y mutation may affect the binding of N1 neuraminidase, not only to oseltamivir but to peramivir [14]. Further study is necessary to investigate clinical impact correlating increased IC_{50} values.

In conclusion, A(H1N1)pdm09, H3N2, and B were prevalent in the 2010–2011 season in Japan, with A(H1N1)pdm09 being dominant. Of the A(H1N1)pdm09 isolates, two of 269 displayed high IC_{50} values for oseltamivir and peramivir. No isolates displayed significantly high IC_{50} values for zanamivir and laninamivir.

Acknowledgments We thank the following doctors for participating in this study: Dr. Yuriko Tarukawa (Tarukawa Clinic), Dr. Kouichi Mochizuki (MOCHIZUKI NAIKA clinic), Dr. Yasuo Sato (Sato clinic), Dr. Norio Yamaguchi (Yamaguchi medical and respiratory clinic), Dr. Tadahiko Ogasawara and Dr. Tsuneo Inoue (Medical Corporation Sai Tadayoshi Kai SAIKATSU CLINIC), Dr. Hiroshi Ukai (UKAI CLINIC), Dr. Nobuo Hirotsu (Hirotsu Clinic), Dr. Takashi Kawashima (Kawashima Medical Clinic), Dr. Naoki Kawai (Kawai Clinic), Dr. Satoshi Yamauchi (Yamauchi Clinic), Dr. Jun Ogawa and Dr. Kyosuke Kaji (Dr. Handa's medical office), Dr. Kunio Kondo and Dr. Yasuo Ontachi (Kondou clinic), Dr. Yutaka Wakasa (Wakasa medical clinic), Dr. Norio Iwaki (Iwaki's Clinic), Dr. Ken-ichi Doniwa (Clinic Doniwa), Dr. Shinro Matsuura (Matsuura Clinic), Dr. Kiyoshi Nishikawa (Nishikawa clinic), Dr. Osame Tanaka (Tokujikai Tanaka clinic), Dr. Hiroko Kondo, Dr. Atsuko Nabeshima (Haradoi Hospital), Dr. Miki Hirata and Dr. Yasuhiko Hirata (Hirata Medical Clinic), Dr. Keisuke Egashira, Dr. Shunsuke Akimitsu, Dr. Keita Tatsushima, Dr. Masaaki Chinen, Dr. Yoshinori Nishimoto, and Dr. Masashi Miyazaki (Sakura Hospital), Dr. Tetsunari Maeda (Sakura Clinic), and Dr. Hiroko Kondo, Dr. Atsuko Nabeshima (Haradoi Hospital).

References

1. Transmission dynamics and impact of pandemic influenza A (H1N1) 2009 virus. Wkly Epidemiol Rec. 2009;46:481–4. http://www.who.int/wer/2009/wer8446.pdf.
2. Kawai N, Ikematsu H, Iwaki N, Satoh I, Kawashima T, Maeda T, et al. Factors influencing the effectiveness of oseltamivir and amantadine for the treatment of influenza: a multicenter study from Japan of the 2002–2003 influenza season. Clin Infect Dis. 2005;40(9):1309–16.
3. Kawai N, Ikematsu H, Iwaki N, Maeda T, Satoh I, Hirotsu N, et al. A comparison of the effectiveness of oseltamivir for the treatment of influenza A and influenza B: a Japanese multicenter study of the 2003–2004 and 2004–2005 influenza seasons. Clin Infect Dis. 2006;43(4):439–44.
4. Kawai N, Ikematsu H, Iwaki N, Kawashima T, Maeda T, Mitsuoka S, et al. Longer virus shedding in influenza B than in influenza A among outpatients treated with oseltamivir. J Infect. 2007;55:267–72.
5. Lackenby A, Hungnes O, Dudman SG, Meijer A, Paget WJ, Hay AJ, et al. Emergence of resistance to oseltamivir among influenza A(H1N1) viruses in Europe. Euro Surveill. 2008;13:1–2.
6. Influenza A virus resistance to oseltamivir and other antiviral medicines 4 June 2009. http://www.who.int/csr/disease/influenza/2008-9nhemisummaryreport/en/.
7. Gubareva LV, Webster RG, Hayden FG. Comparison of the activities of zanamivir, oseltamivir, and RWJ-270201 against clinical isolates of influenza virus and neuraminidase inhibitor-resistant variants. Antimicrob Agents Chemother. 2001;45:3403–8.
8. Kawai N, Ikematsu H, Iwaki N, Kondou K, Hirotsu N, Kawashima T, et al. Clinical effectiveness of oseltamivir for influenza A (H1N1) virus with H274Y neuraminidase mutation. J Infect. 2009;59(3):207–12.
9. Kawai N, Ikematsu H, Hirotsu N, Maeda T, Kawashima T, Tanaka O, et al. Clinical effectiveness of oseltamivir and zanamivir for treatment of influenza A virus subtype H1N1 with the H274Y mutation: a Japanese multicenter study of the 2007–2008 and 2008–2009 influenza seasons. Clin Infect Dis. 2009;49(12):1828–35.
10. Nguyen HT, Sheu TG, Mishin VP, Klimov AI, Gubareva LV. Assessment of pandemic and seasonal influenza A (H1N1) virus susceptibility to neuraminidase inhibitors in three enzyme activity inhibition assays. Antimicrob Agents Chemother. 2010;54:3671–7.
11. Stockton J, Ellis JS, Saville M, Clewley JP, Zambon MC. Multiplex PCR for typing and subtyping influenza and respiratory syncytial viruses. J Clin Microbiol. 1998;36:2990–5.
12. Yamashita M, Tomozawa T, Kakuta M, Tokumitsu A, Nasu H, Kubo S. CS-8958, a prodrug of the new neuraminidase inhibitor R-125489, shows long-acting anti-influenza virus activity. Antimicrob Agents Chemother. 2008;53:186–92.
13. Memoli MJ, Hrabal RJ, Hassantoufighi A, Eichelberger MC, Taubenberger JK. Rapid selection of oseltamivir- and peramivir-resistant pandemic H1N1 virus during therapy in 2 immunocompromised hosts. Clin Infect Dis. 2010;50(9):1252–5.
14. Collins PJ, Haire LF, Lin YP, Liu J, Russell RJ, Walker PA, et al. Crystal structures of oseltamivir-resistant influenza virus neuraminidase mutants. Nature. 2008;453:1258–61.

Genetic Characterization of Human Influenza Viruses in the Pandemic (2009–2010) and Post-Pandemic (2010–2011) Periods in Japan

Isolde C. Dapat[1]*, Clyde Dapat[1], Tatiana Baranovich[1¤a], Yasushi Suzuki[1¤b], Hiroki Kondo[1], Yugo Shobugawa[1], Reiko Saito[1], Hiroshi Suzuki[2], the Japanese Influenza Collaborative Study Group[¶]

1 Division of International Health (Public Health), Graduate School of Medical and Dental Sciences, Niigata University, Niigata, Japan, **2** School of Nursing, Niigata Seiryo University, Niigata, Japan

Abstract

Background: Pandemic influenza A(H1N1) 2009 virus was first detected in Japan in May 2009 and continued to circulate in the 2010–2011 season. This study aims to characterize human influenza viruses circulating in Japan in the pandemic and post-pandemic periods and to determine the prevalence of antiviral-resistant viruses.

Methods: Respiratory specimens were collected from patients with influenza-like illness on their first visit at outpatient clinics during the 2009–2010 and 2010–2011 influenza seasons. Cycling probe real-time PCR assays were performed to screen for antiviral-resistant strains. Sequencing and phylogenetic analysis of the HA and NA genes were done to characterize circulating strains.

Results and Conclusion: In the pandemic period (2009–2010), the pandemic influenza A(H1N1) 2009 virus was the only circulating strain isolated. None of the 601 A(H1N1)pdm09 virus isolates had the H275Y substitution in NA (oseltamivir resistance) while 599/601 isolates (99.7%) had the S31N substitution in M2 (amantadine resistance). In the post-pandemic period (2010–2011), cocirculation of different types and subtypes of influenza viruses was observed. Of the 1,278 samples analyzed, 414 (42.6%) were A(H1N1)pdm09, 525 (54.0%) were A(H3N2) and 33 (3.4%) were type-B viruses. Among A(H1N1)pdm09 isolates, 2 (0.5%) were oseltamivir-resistant and all were amantadine-resistant. Among A(H3N2) viruses, 520 (99.0%) were amantadine-resistant. Sequence and phylogenetic analyses of A(H1N1)pdm09 viruses from the post-pandemic period showed further evolution from the pandemic period viruses. For viruses that circulated in 2010–2011, strain predominance varied among prefectures. In Hokkaido, Niigata, Gunma and Nagasaki, A(H3N2) viruses (A/Perth/16/2009-like) were predominant whereas, in Kyoto, Hyogo and Osaka, A(H1N1)pdm09 viruses (A/New_York/10/2009-like) were predominant. Influenza B Victoria(HA)-Yamagata(NA) reassortant viruses (B/Brisbane/60/2008-like) were predominant while a small proportion was in Yamagata lineage. Genetic variants with mutations at antigenic sites were identified in A(H1N1)pdm09, A(H3N2) and type-B viruses in the 2010–2011 season but did not show a change in antigenicity when compared with respective vaccine strains.

Citation: Dapat IC, Dapat C, Baranovich T, Suzuki Y, Kondo H, et al. (2012) Genetic Characterization of Human Influenza Viruses in the Pandemic (2009–2010) and Post-Pandemic (2010–2011) Periods in Japan. PLoS ONE 7(6): e36455. doi:10.1371/journal.pone.0036455

Editor: Man-Seong Park, College of Medicine, Hallym University, Republic of Korea

Received September 26, 2011; **Accepted** April 5, 2012; **Published** June 27, 2012

Copyright: © 2012 Dapat et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was supported by the Health Labour Sciences Research Grant, Research on Emerging and Re-emerging Infectious Diseases (Ministry of Health, Welfare and Labour, Japan). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: sol@med.niigata-u.ac.jp

¤a Current address: St. Jude Children's Research Hospital, Memphis, Tennessee, United States of America
¤b Current address: National Institute of Animal Health, National Agriculture and Food Research Organization, Tsukuba, Ibaraki, Japan

¶ For a list of the members of the Japanese Influenza Collaborative Study Group please see the Acknowledgments section

Introduction

In late March to early April of 2009, a novel influenza virus of swine origin emerged in humans in Mexico and the USA and rapidly spread worldwide, prompting the WHO to declare an influenza pandemic [1–3]. This is the first influenza pandemic since the A(H3N2) Hong Kong pandemic of 1968. The pandemic A(H1N1) 2009 virus [A(H1N1)pdm09] was initially found to be susceptible to neuraminidase inhibitors, oseltamivir and zanamivir, but resistant to amantadine [4]. In the course of the pandemic in the 2009–2010 period, sporadic cases of oseltamivir-resistant strains were detected around the world [5,6]. Monitoring of the antiviral resistance of A(H1N1) viruses is important because of the widespread resistance of seasonal A(H1N1) viruses to oseltamivir beginning in the 2007–2008 season [7,8]. Drug-resistant pandemic A(H1N1) viruses that could acquire the ability to be transmitted efficiently among humans pose a considerable public health concern.

In Japan, the first pandemic influenza case was reported in May 2009 [9]. In mid-June 2009, the pandemic influenza A(H1N1) virus had spread throughout Japan and by mid-July, all 47

prefectures were affected [10]. Phylogenetic analysis of these pandemic stage viruses revealed that the A(H1N1)pdm09 virus had evolved since its first appearance in the country [11]. Sequence analysis of viruses from the very early phase (May 2009) and from the peak phase (October 2009 to January 2010) of the pandemic identified distinct mutations in the HA and NA that clearly differentiate viruses from these two time periods [12].

In this study, we described the circulation patterns and genetic characteristics of viruses that circulated during the pandemic and post-pandemic periods. We focused on the comparison of pandemic influenza A(H1N1) viruses collected in Japan in the 2009–2010 and 2010–2011 seasons. In addition, we performed genetic analysis on A(H3N2) and influenza B viruses that cocirculated with the A(H1N1)pdm09 viruses in the 2010–2011 season.

Results

Clinical Background of Patients

Seven hundred thirty three (733) patients with influenza-like illness who visited outpatient clinics in six prefectures in Japan (Fukushima, Gunma, Niigata, Kyoto, Hyogo and Nagasaki) between July 2009 and February 2010, and 1,278 patients in seven prefectures (Hokkaido, Niigata, Gunma, Kyoto, Hyogo, Osaka and Nagasaki) from December 2010 to March 2011 participated in our study. Among these patients, 4 from the 2009–2010 period and 14 from the 2010–2011 period were hospitalized after the initial assessment of the severity of infection at the outpatient clinic (data not shown). All of the hospitalized patients from the two periods fully recovered. There were no patients with lethal infection.

Laboratory Surveillance of Influenza Viruses

Of the 733 respiratory specimens that were collected from patients with influenza-like illness in the 2009–2010 season, 601 A(H1N1)pdm09 viruses (85.4%) were isolated, as confirmed by the real-time PCR assays (Table 1). A part of the samples were verified by hemagglutination inhibition (HI) test. A(H1N1)pdm09 influenza virus activity peaked in November 2009 (week 47) (Figure 1) which was 2–3 months earlier than in previous years. No other type or subtype of influenza viruses was detected during this period. The pandemic A(H1N1) 2009 virus had completely replaced the seasonal A(H1N1) virus in the areas studied within the surveillance period.

Of the 1,278 respiratory specimens that were collected in the 2010–2011 season 972 (76.1%) viruses were isolated. There was cocirculation of different types and subtypes of influenza viruses in this season and were distributed as follows: 414 A(H1N1)pdm09 (42.6%), 525 A(H3N2) (54.0%) and 33 influenza B (3.4%) viruses (Table 1). There were no influenza B viruses detected in Hokkaido, Gunma and Osaka prefectures. Pandemic A(H1N1) 2009 virus activity peaked in January (weeks 3 and 4) while A(H3N2) virus activity peaked in February (week 6) (Figure 1).

There was variability in the influenza virus subtype predominance in each prefecture. A(H3N2) viruses were predominant in Hokkaido, Niigata and Gunma prefectures in the northern area of Japan, as well as in Nagasaki in the south. A(H1N1)pdm09 viruses were predominant in prefectures in the Kansai area of western Japan: Kyoto, Hyogo and Osaka (Table 1, Figure 2).

Among influenza B viruses, 31 out of 33 (93.9%) isolates belong to the Victoria lineage and 2 out of 33 (6.1%) isolates belong to the Yamagata lineage, according to cycling probe real time PCR results (Table 1).

Detection of H275Y Mutation in the NA

All of the 601 A(H1N1)pdm09 viruses from the 2009–2010 period showed possession of H275 (wild-type) in the neuraminidase (NA) by screening with cycling probe real-time PCR and/or by genetic sequencing. These results suggested possible susceptibility to oseltamivir among the collected specimens.

In the 2010–2011 season, 2 out of 414 (0.5%) isolates harbor the H275Y mutation in NA as shown by cycling probe assay and sequencing. These viruses were from primary respiratory specimens of patients with no prior treatment of oseltamivir.

Detection of S31N Mutation in the M2

All of the 1,015 A(H1N1)pdm09 isolates in the two seasons were tested for the presence of M2-S31N substitution that confers resistance to amantadine using the cycling probe real time PCR method. All but two viruses possessed the M2-S31N change: A/Nagasaki/09N079/2009 and A/Kyoto/09K084/2009 had serine (AGT) at position 31 (as confirmed by sequencing of the transmembrane domain of the M2 gene) suggesting susceptibility to amantadine. All A(H1N1)pdm09 viruses from the 2010–2011 season harbor the S31N mutation in M2 (Table 1).

Among the A(H3N2) isolates, 520/525 (99.0%) had the M2-S31N substitution according to cycling probe real time PCR results (Table 1), suggesting resistance to amantadine.

Phylogenetic Analysis

a. Pandemic influenza A (H1N1) 2009. Sequence and phylogenetic analyses of the hemagglutinin (HA) and neuraminidase (NA) genes of 81 pandemic influenza A(H1N1) viruses from the 2009–2010 season and 55 from the 2010–2011 season were performed.

In the 2009–2010 season, 80 out of 81 (98.8%) isolates had the S203T mutation in the HA that characterizes cluster 2 viruses [13]. These viruses were A/New York/10/2009-like (Figure 3). One isolate, A/Nagasaki/09N083/2009, had serine (S) at amino acid position 203, belonged to cluster 1 and was related to the vaccine strain A/California/07/2009. On the other hand, all isolates had the cluster 2-characteristic N248D amino acid substitution in the NA. In the 2010–2011 season, HA and NA phylogenies showed that all 55 A(H1N1)pdm09 viruses belonged to cluster 2 (Figure 3).

A(H1N1)pdm09 viruses from the 2009–2010 season were located near the trunk of the HA phylogenetic tree. Comparison of the HA gene of the A(H1N1)pdm09 viruses with the vaccine strain (A/California/7/2009) showed 2–14 amino acid mutations. Among these mutations, 5 are located in antigenic sites Ca (G170E, S203T, R205K and D222E) and Cb (L70F) [14] (Figure 3A, Figure 4A). A(H1N1)pdm09 viruses from the 2010–2011 season exhibited additional amino acid changes in the HA from the previous season's strains. Amino acid substitutions S185T and A197T were observed in fifty-three (53) isolates analyzed. Amino acid mutations A134T, A141S, S183P and I295V were detected in two (2) isolates. When compared with the vaccine strain, 4–14 amino acid mutations were identified. Several of these mutations are localized in antigenic sites Ca (A141S, G140E, I166V and E235K), Sa (K153T, K160M, K163N and K163T) and Sb (S185T and A186T) (Figure 3A, Figure 4A). HI test with selected strains showed similar antigenicity with the vaccine strain despite several amino acid changes at putative antigenic sites (Table S1).

An amino acid change from aspartic acid (D) to glutamic acid (E) was observed at residue 222 in the HA in 6 isolates from the 2009–2010 season. However, this amino acid substitution was not found in 2010–2011 isolates. Glycine (G)

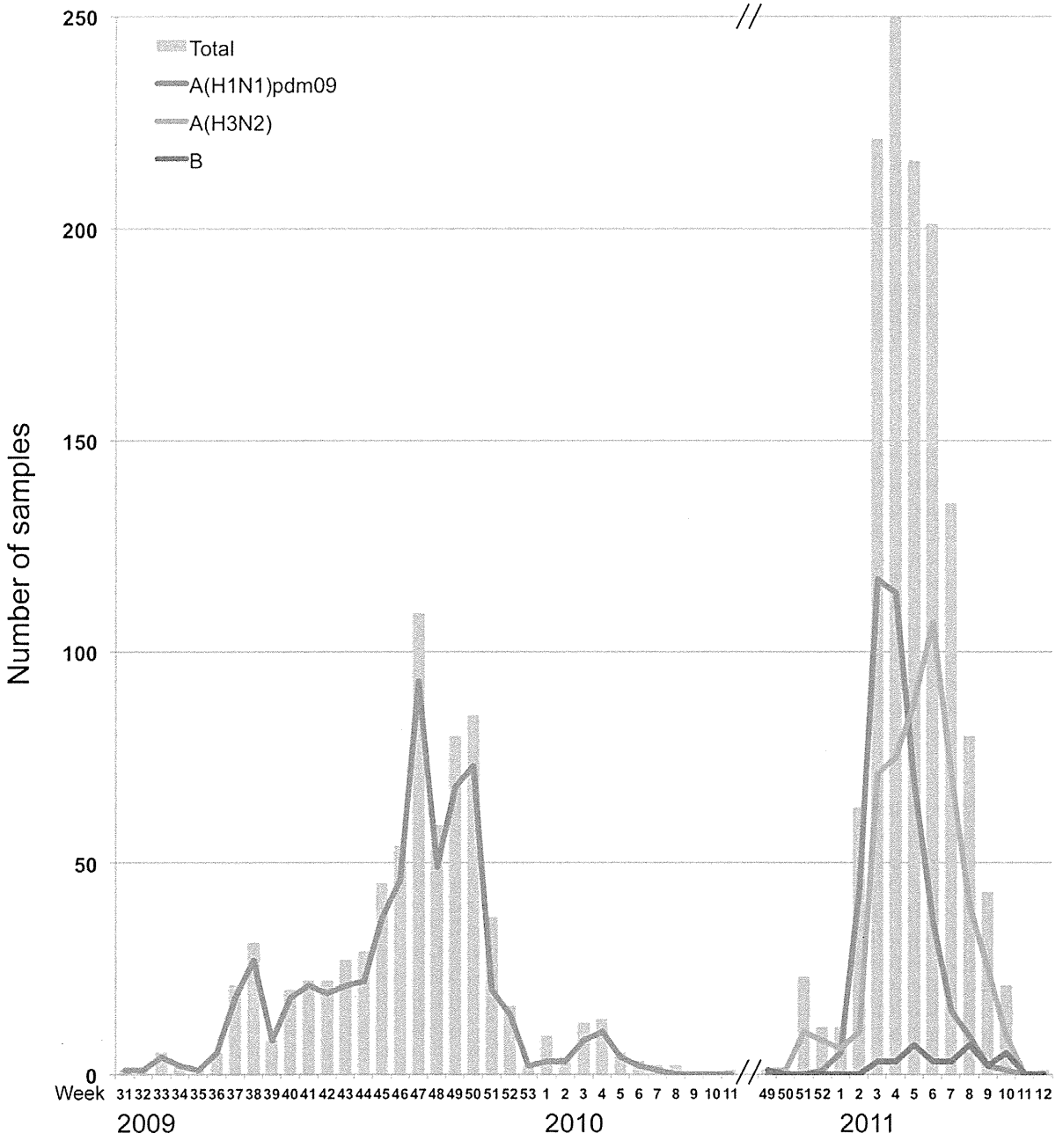

Figure 1. Number of influenza cases per week that were tested positive using the cycling probe real-time PCR method in the 2009–2010 and 2010–2011 seasons. The figure shows two influenza periods, the pandemic period (2009–2010) and the post-pandemic period (2010–2011). In 2009–2010, only influenza A(H1N1)pdm09 viruses were detected and its activity peaked at week 47 (November). In 2010–2011, influenza A(H1N1)pdm09, A(H3N2), and type-B viruses were detected. Activity of A(H1N1)pdm09 viruses peaked at week 3 (January) while activity of A(H3N2) viruses peaked at week 6 (February). Sporadic cases of influenza B were observed.
doi:10.1371/journal.pone.0036455.g001

or asparagine (N) mutation at amino acid residue 222, which were previously reported to be associated with severe illness [15,16], were not observed among the isolates from both seasons.

The NA phylogenetic tree was generally congruent with that of the HA (Figure 3B). Comparison of the NA gene of A(H1N1)pdm09 viruses with the vaccine strain showed 2–7 amino acid susbstitutions among isolates in the 2009–2010 season and 3–12 substitutions in the 2010–2011 season. The 2010–2011 season viruses were closely related to a strain from the previous season, A/Kyoto/09K033/2009, and had the characteristic amino acid substitutions V241I, N44S and N369K [17] (Figure 3B, Figure 5A). No mutations were found in the antigenic sites.

Two (2) oseltamivir resistant strains with the H275Y mutation in the NA collected in 2010–2011 were located in the same branch in the NA phylogeny. However, these two strains were found in different branches in the HA phylogeny (Figure 3).

Two (2) amantadine-sensitive strains collected in 2009–2010 were distributed in different branches in both HA and NA

Table 1. Detection of influenza virus type, subtype and antiviral-resistance by cycling probe real time PCR.

Prefecture	2009–2010 season				2010–2011 season						
	No. of samples	Influenza A			No. of samples	Influenza A					Influenza B
		A(H1N1)pdm09	OsR*	AmR**		A(H1N1)pdm09	OsR*	AmR**	A(H3N2)	AmR**	
Hokkaido	-	-	-	-	80	6	0	6	38	38	0
Fukushima	93	51	0	51	-	-	-	-	-	-	-
Gunma	31	27	0	27	46	5	0	5	35	35	0
Niigata	100	74	0	74	555	65	0	65	295	295	15
Kyoto	306	295	0	294	328	217	1	217	59	54	14
Osaka	-	-	-	-	50	21	0	21	6	6	0
Hyogo	66	60	0	60	99	59	1	59	33	33	3
Nagasaki	137	94	0	93	120	41	0	41	59	59	1
TOTAL	733	601	0 (0.0%)[a]	599 (99.7%)[a]	1278	414	2 (0.5%)[a]	414 (100%)[a]	525	520 (99.0%)[a]	33

*oseltamivir-resistant (OsR): H275Y mutation in NA.
**amantadine-resistant (AmR): S31N mutation in M2.
"-" no samples collected.
[a]Percentage of antiviral resistant viruses in each season.
doi:10.1371/journal.pone.0036455.t001

phylogenies (Figure 3). In the M gene phylogeny, these two strains clustered with the A(H1N1)pdm09 clade but not with the European avian-like H1N1, triple reassortant H1N2 or classical swine clades, suggesting no evidence of reassortment from other swine lineages (Figure S1).

b. Influenza A(H3N2). The hemagglutinin (HA) and neuraminidase (NA) genes of 71 A(H3N2) viruses from the 2010–2011 season were analyzed. The HA and NA phylogenies showed that 48 isolates belonged to the A/Perth/16/2009 (Perth16) clade and 23 isolates belonged to the A/Victoria/208/2009 (Vic208) clade (Figure 6). When compared with the vaccine strain, A/Perth/16/2009, A(H3N2) isolates had 2–17 amino acid mutations in the HA, 9 of which are found in antigenic sites A (N144K), B (P162S), C (E50K, D53N and E280A/S/T) and E (I260M and R261Q) [18] (Figure 4B, Figure 6A). The Perth16 clade was characterized by 5 amino acid mutations at residues 62, 144, 162, 260 and 261 when compared with the vaccine strain. Of these, 4 are found at antigenic sites A (N144K), B (P162S) and E (I260M and R261Q). Selected Perth16 clade viruses showed similar antigenicity to the vaccine strain in the HI test (Table S2).The Vic208 clade was characterized by the amino acid change T212A located in antigenic site C. Interestingly, geographical and temporal clustering was observed

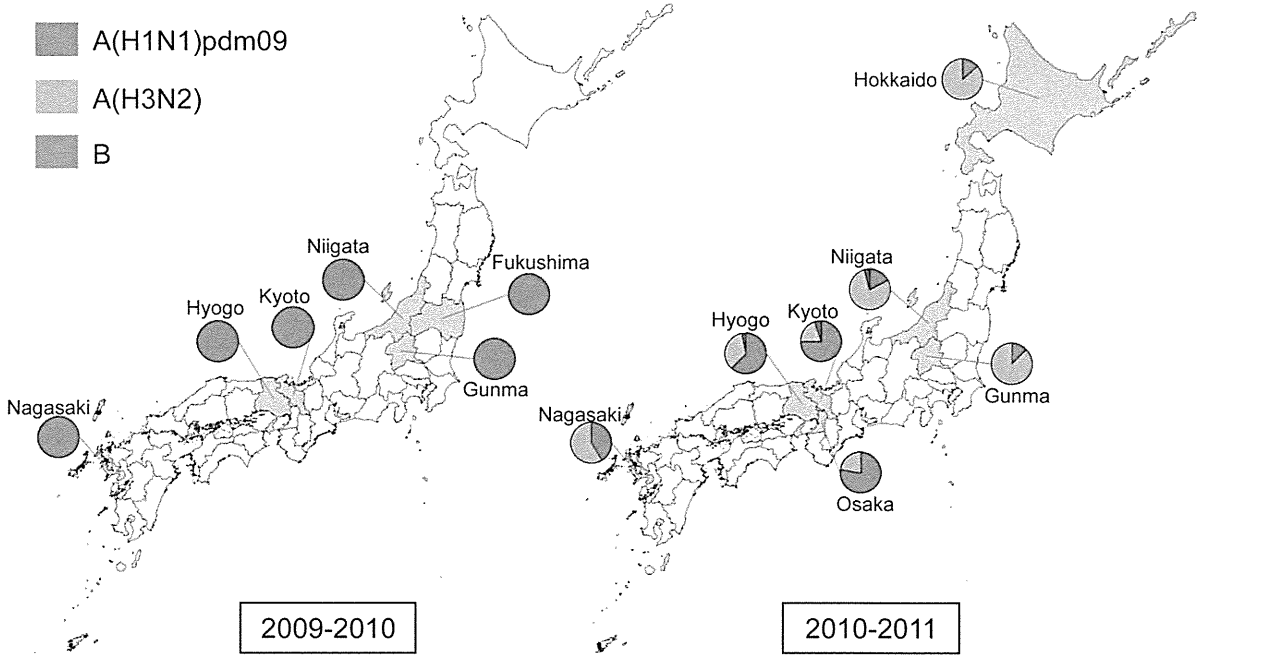

Figure 2. Geographic distribution of influenza isolates in the 2009–2010 and 2010–2011 seasons in Japan.
doi:10.1371/journal.pone.0036455.g002

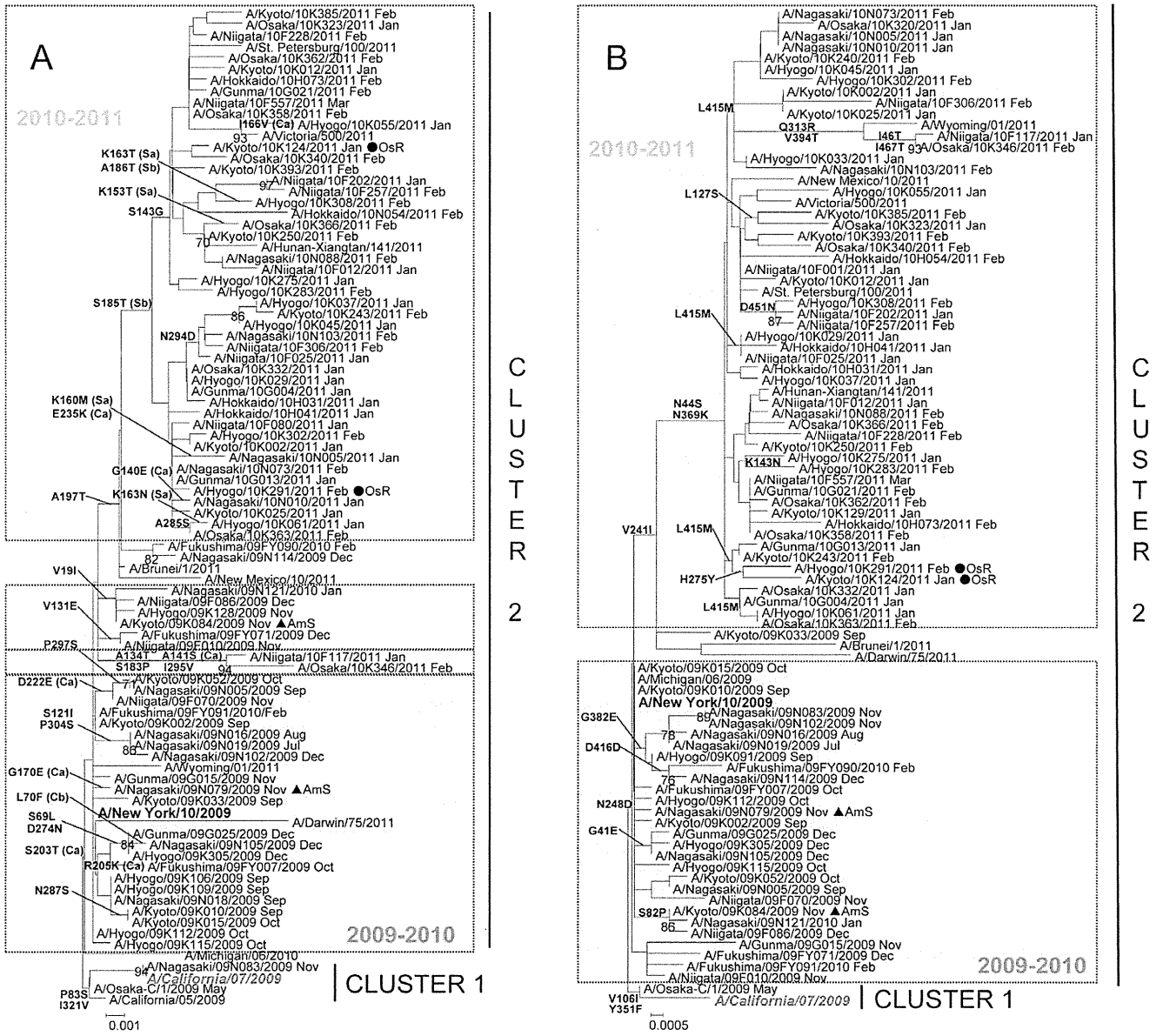

Figure 3. Phylogenetic analysis of the A) HA1 fragment of hemagglutinin, HA gene (829nt) and B) neuraminidase, NA gene (1,413nt) of A(H1N1)pdm isolates. Trees were constructed using the Neighbor-Joining method. Numbers at the nodes indicate confidence levels of bootstrap analysis with 1,000 replicates as percentage value. Amino acid substitutions that characterized a particular branch are indicated on the left side node. Vaccine strains are italicized and in red. Reference strains are boldfaced. Sequences from 2009–2010 are in pink and sequences from 2010–2011 are in blue. Oseltamivir-resistant strains (OsR) are indicated with filled circles (●) and amantadine-sensitive strains (AmS) are indicated with filled triangles (▲).
doi:10.1371/journal.pone.0036455.g003

with A(H3N2) viruses. Most of Perth16 clade viruses circulated in Hokkaido, Gunma, Niigata and Nagasaki from December 2010, while Vic208 clade viruses was observed only in the Kansai area (Kyoto, Osaka and Hyogo) after January 2011.

Mutations in the NA of A(H3N2) viruses ranged from 4–17 amino acid residues when compared with the vaccine strain (Figure 6B). Of these, 4 are located in antigenic sites F' (L338F), I' (K369T) and K' (R400K and N402D) [19] (Figure 5B, Figure 6B). Amino acid changes D127N, I307M, L338F and N342D are unique to the Perth16 clade. The L338F substitution is located in antigenic site F'. Amino acid substitutions K369T, I464L and S367N are unique to the Vic208 clade strains, with K369T found in antigenic site I'.

c. Influenza B viruses. Genetic analysis of the HA and NA sequences of 29 influenza B viruses was performed. The HA phylogeny showed that 27 viruses belonged to the Victoria lineage; 26 viruses were closely related to the vaccine strain, B/Brisbane/60/2008 (Brisbane60) and 1 virus (B/Niigata/10F478/2011) was closely related to an earlier vaccine strain, B/Malaysia/2506/2004. The remaining 2 viruses belonged to the Yamagata-lineage and were B/Bangladesh/3333/2007-like (Bangladesh3333) (Figure 7A). Within the Brisbane60 clade of the Victoria lineage, 6 amino acid substitutions were identified when compared with the vaccine strain; 4 of which are found in antigenic sites A (A127T and I146V), B (K165N and K209N) [20] (Figure 4C). The amino acid change I146V was found in all Brisbane60 clade

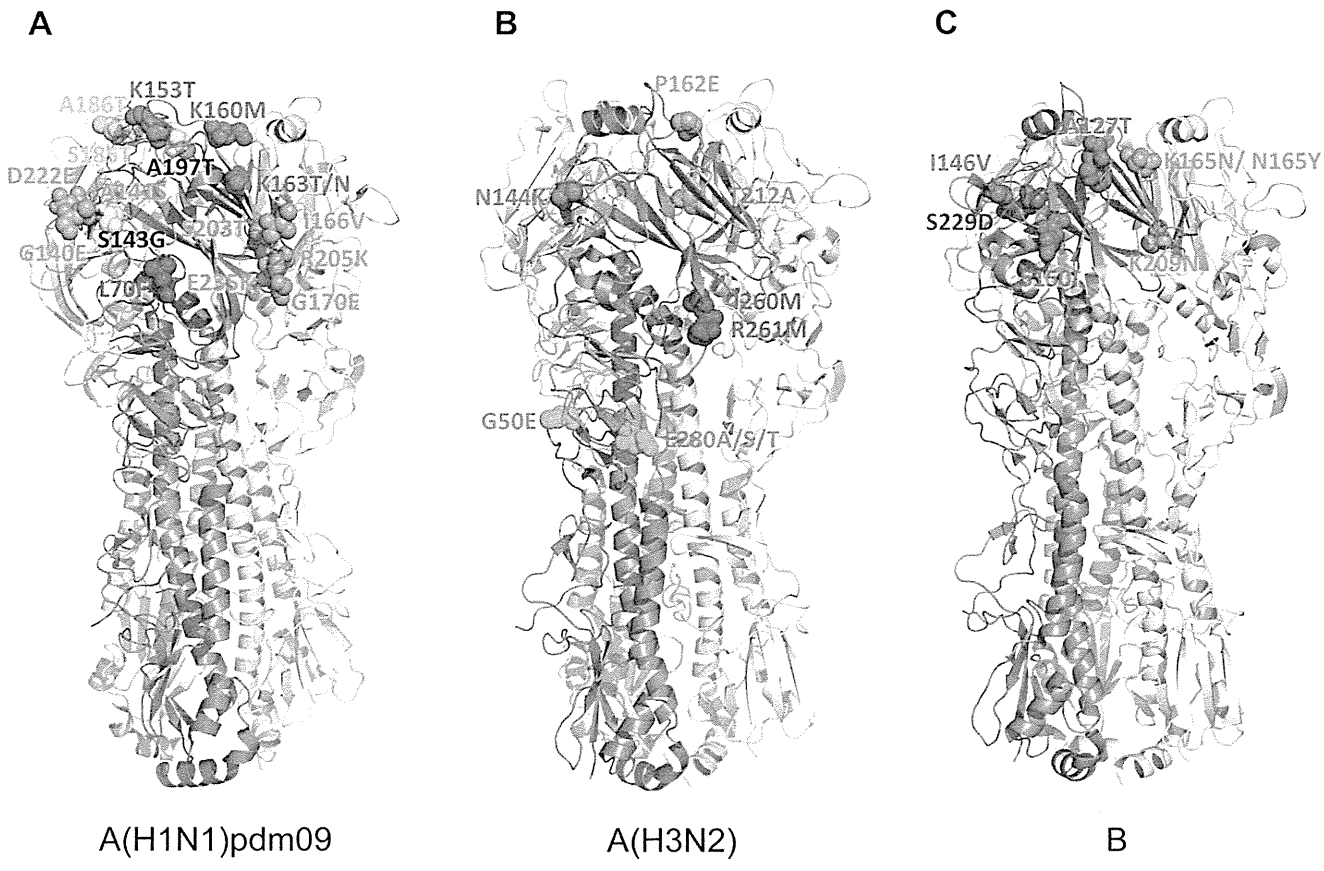

Figure 4. Observed mutations in the antigenic sites of HA of influenza virus isolates in Japan, 2009–2010 and 2010–2011. Three-dimensional structures of trimeric HA were downloaded from the Protein Data Bank (RCSB PDB, http://www.pdb.org) [44] and visualized using PyMol (http:www.pymol.org). (A) The amino acid differences in the antigenic sites of HA between Japanese A(H1N1)pdm09 isolates and vaccine strain, A/California/07/2009 were compared. Amino acid substitutions at G140E, A141S, I166V, G170E, S203T, R203T, D222E, E235K are located in antigenic site Ca (orange); L70F is located in antigenic site Cb (blue); K153T, K160M, K163T/N in antigenic site Sa (magenta); and S185T, A186T in antigenic site Sb (cyan). Amino acid changes outside the antigenic sites are shown in yellow. PDB entry: 3LZG. (B) HA antigenic site mutations between Japanese A(H3N2) isolates and vaccine strain, A/Perth/16/2009 were compared. N144K mutation is localized in antigenic site A (red); P162S in antigenic site B (orange); G50E/K. T212A, and E280A/S/T are localized in antigenic site C (green); I260M and R261Q are located in antigenic site E (blue). PDB entry: 1MQL (C) Amino acid substitutions in the HA antigenic sites of influenza B isolates in Japan and vaccine strain, B/Brisbane/60/2008 were compared. Mutations at A127T, V146I, and S150I are localized at antigenic site A (red); N165K/Y and K209N are located in antigenic site B (orange); and S229D is located in antigenic site D (violet). PDB entry: 2RFT.
doi:10.1371/journal.pone.0036455.g004

viruses. Within the Bangladesh3333 clade of the Yamagata lineage, 8 amino acid changes were found when compared with the vaccine strain, B/Florida/4/2006. Three of these substitutions are located in antigenic sites A (S150I), B (N165Y) and D (S229D) (Figure 4C). Viruses from both clades showed compatible antigenicities when compared with their respective vaccine strains (Table S3).

The NA phylogeny showed that all viruses belonged to the Yamagata-lineage but the clustering into two distinct groups was similar to that of the HA (Figure 7B). Within the Brisbane60 clade, 13 amino acid changes were observed; of which, 2 are found in antigenic sites F' (N329D) and G (N340D) (Figure 5C). Within the Bangladesh3333 clade, 8 amino acid substitutions were identified wherein 1 amino acid change is located in the antigenic site G' (D340N) (Figure 5C, Figure 7B).

Discussion

The pandemic influenza A(H1N1) virus first appeared in Japan in May 2009 and reached the pandemic stage in June 2009 [9]. In the course of the surveillance study we conducted during the pandemic period, A(H1N1)pdm09 viruses were the only circulating strains detected. The seasonal A(H1N1) virus that was predominant until the 2008–2009 season was not detected in Japan after week 36 of 2009 [21]. In August 2010, the WHO announced that the A(H1N1)pdm09 virus had moved into the post-pandemic period. It continued to circulate worldwide in the 2010–2011 season. In contrast to the pattern we observed during the pandemic period, the A(H1N1)pdm09 virus cocirculated with other influenza viruses, namely A(H3N2) and type-B viruses. The percentage of A(H1N1)pdm09 viruses that were isolated in our surveillance study went from 100% during the pandemic period (2009–2010) to 43% in the post-pandemic period (2010–2011). The decrease in the number of clinical cases may be attributed to an increase in antibody levels against the A(H1N1)pdm09 virus in the community [22,23]. This is supported by the results of the sero-surveillance studies conducted by the Infectious Disease Surveillance Center in 2009 and in 2010 that showed a substantial increase in the antibody levels of those surveyed in 2010, reaching a high prevalence rate of over 50% among school-aged children, when compared with the antibody levels in 2009 (prevalence rate

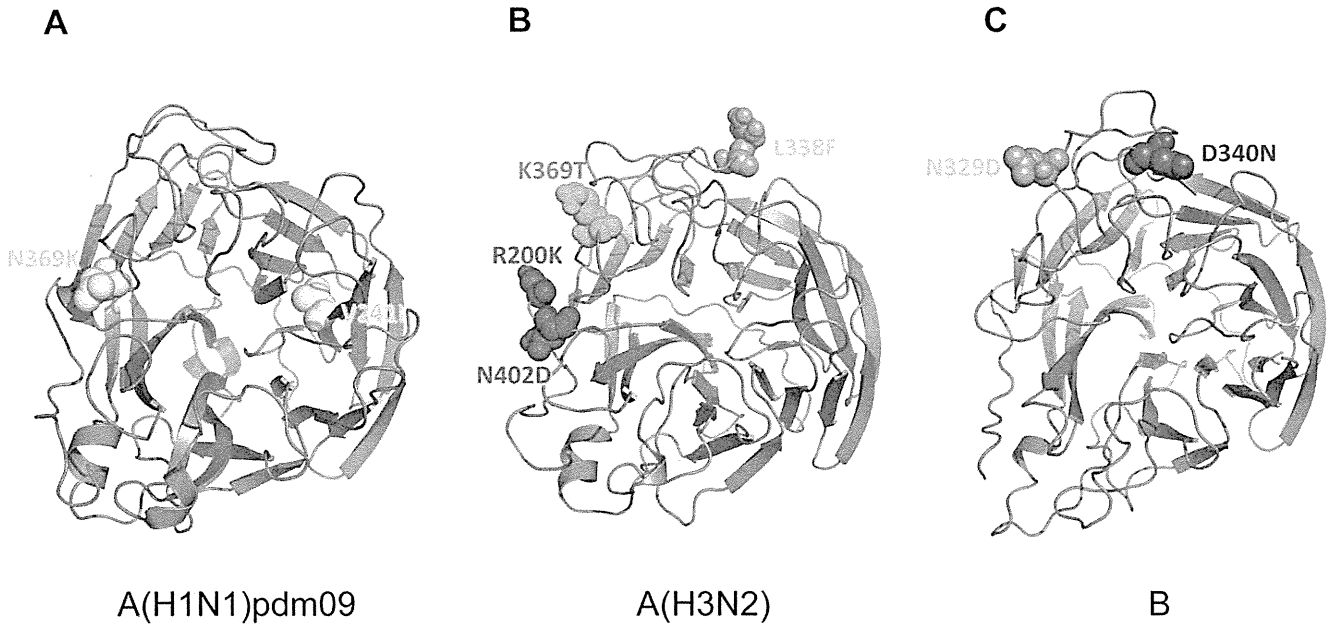

Figure 5. Amino acid mutations differences in the NA of influenza virus isolates in Japan, 2009–2010 and 2010–2011. Three-dimensional structures of monomeric NA were downloaded from the Protein Data Bank (RCSB PDB, http://www.pdb.org) [44] and visualized using PyMol (http:www.pymol.org). The top view of the NA is shown. (A) The amino acid differences between A(H1N1)pdm09 isolates in Japan and vaccine strain, A/California/07/2009 were compared. Amino acid substitutions V241I and N369K are shown in yellow. These amino acid changes are located outside the antigenic sites but are phylogenetically relevant. PDB entry: 3NSS. (B) Antigenic site mutations between Japanese A(H3N2) isolates and vaccine strain, A/Perth/16/2009 were compared. L338F mutation is located in antigenic site F′ (olive); K369T is localized in antigenic site I′; and R400K and N402D are located in antigenic site K′. PDB entry: 1IVG (C) Amino acid substitutions in the NA antigenic sites of Japanese influenza B isolates and vaccine strain, B/Brisbane/60/2008 were compared. Mutations at N329D is localized at antigenic site F′ (olive); and D340N/D is located in antigenic site G′ (violet). PDB entry: 1INF.
doi:10.1371/journal.pone.0036455.g005

of 5–20%) (http://idsc.nih.go.jp/yosoku/Flu/2010Flu/Flu10_2.html, [Japanese]).

Influenza A(H3N2) was the predominant influenza type-A virus that caused illness in the 2010–2011 season in our study. Strain predominance varied among prefectures but geographic clustering was evident. Prefectures in northern Japan had 4 to 7 times more A(H3N2) viruses detected than A(H1N1)pdm09 viruses. Prefectures in the Kansai area (Kyoto, Hyogo and Osaka) had about 2 to 4 times more A(H1N1)pdm09 viruses detected than A(H3N2) viruses. Influenza virus peak activity also varied among the type-A viruses. The A(H1N1)pdm09 virus activity peaked in late January whereas A(H3N2) virus activity peaked in mid-February. We could not assess the peak of influenza B due to the termination of the study in early March. According to the National Influenza Surveillance in Japan, more influenza B viruses than influenza A viruses were detected after week 12 until week 20 (http://idsc.nih.go.jp/iasr/influ-e.html) in Japan.

Characteristics of A(H1N1)pdm09 Viruses

In the first year of pandemic (2009–2010), we did not detect any oseltamivir-resistant A(H1N1)pdm09 viruses. In the following season, we detected two (2) A(H1N1)pdm09 viruses (0.5% prevalence) that possessed the H275Y substitution in the NA. These two isolates came from patients who had not received oseltamivir treatment, live in different areas and were infected at different times. There was no epidemiological link established between the two cases. These naturally-occurring oseltamivir-resistant A(H1N1)pdm09 viruses in untreated patients were previously reported in Hong Kong and Vietnam [24,25]. The reported oseltamivir-resistant A(H1N1)pdm09 viruses in Japan in the 2009–2010 season mostly came from patients who received oseltamivir as treatment and as prophylaxis, which suggested sporadic emergence from oseltamivir-sensitive A(H1N1)pdm09 viruses due to selective drug pressure [26]. The two oseltamivir-resistant A(H1N1)pdm09 strains in this study showed a clustering in the NA phylogeny due to the H275Y amino acid substitution, but not in the HA phylogeny. In contrast, the oseltamivir-resistant seasonal A(H1N1) viruses isolated in the 2008–2009 season showed a clustering in both the HA and NA phylogenies with signature amino acid changes other than the H275Y mutation in Japan [8]. The low prevalence of oseltamivir-resistant A(H1N1)pdm09 viruses in this study suggests limited community transmission.

The A(H1N1)pdm09 virus contains the M gene of the Eurasian swine lineage (originally derived from an avian influenza virus) and has the genetic marker (S31N in M2) for resistance to amantadine [27]. In this study, two A(H1N1)pdm09 viruses from the 2009–2010 period have serine (S) at residue 31 of the M2. These viruses were susceptible to amantadine based on the phenotypic assay $TCID_{50}$ (results not shown). DNA sequencing of the M gene of these two viruses showed that the sensitivity was due to a spontaneous mutation in the M2 segment and was not due to a reassortment with an amantadine-sensitive gene segment. From a CDC report [28], it can be inferred that 0.2% (4/1899) of A(H1N1)pdm09 viruses tested were also sensitive to amantadine which suggests that these rare amantadine-sensitive viruses were in circulation in the 2009–2010 season. However, in the following season amantadine-sensitive A(H1N1)pdm09 viruses were not detected in our study.

Based on the HA and NA sequence data of viruses collected within the pandemic period from July 2009 to February 2010,

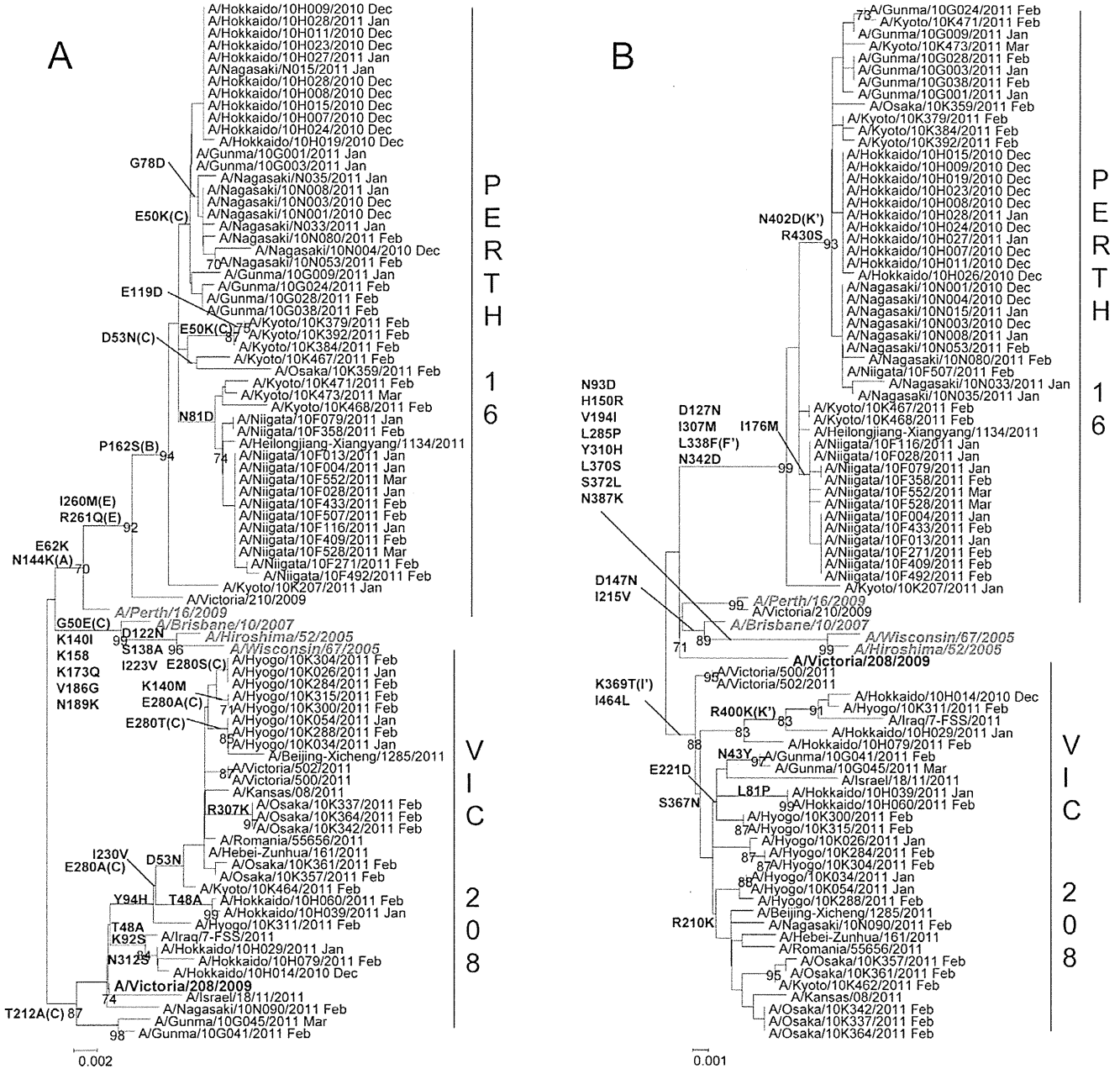

Figure 6. Phylogenetic analysis of the A) HA1 fragment of hemagglutinin, HA gene (954nt) and B) neuraminidase, NA gene (1,388nt) of influenza A(H3N2) isolates. Trees were constructed using the Neighbor-Joining method. Numbers at the nodes indicate confidence levels of bootstrap analysis with 1,000 replicates as percentage value. Amino acid substitutions that characterized a particular branch are indicated on the left side node. Vaccine strains are italicized and in red. Reference strains are boldfaced.
doi:10.1371/journal.pone.0036455.g006

A(H1N1)pdm09 viruses are closely related to each other and to the vaccine strain, A/California/07/2009 and belong to cluster 2. The signature amino acid changes, HA-S203T and NA-N248D, of cluster 2 viruses that differentiate it from early pandemic cluster 1 viruses continued to persist throughout the duration of the pandemic and into the 2010–2011 season. One isolate, A/Nagasaki/09N083/2009, collected in November 2009 has a cluster 1 HA but a cluster 2 NA. This virus belongs to cluster 1.2 which mostly contained viruses from Japan isolated during the early phase of the pandemic [13].

Sequence analysis of A(H1N1)pdm09 viruses of the 2010–2011 season showed further evolution from viruses of the 2009–2010 season. These viruses were closely related to the previous season strains, A/Nagasaki/09N114/2009 and A/Fukushima/09FY090/2010. Viruses with the amino acid substitutions S185T and A197T had the greatest expansion and geographic spread. Structural analysis showed that S185T is found in the antigenic site Sb, which is located on the globular head of the HA (Figure 4A). The amino acid substitution A197T is found in almost all of the viruses from the 2010–2011 season and is located next to the antigenic site Sb.