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

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 <0·05 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, $P < 0·001$) and for A(H3N2) and B in the 2010–2011 season (19·2 ± 19·5 years, $P < 0·01$, and 14·9 ± 11·9 years, $P < 0·001$, 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, $P < 0·05$) and in A(H3N2) and B (39·0 ± 0·7°C, $P < 0·01$ and 38·9 ± 0·5°C, $P < 0·001$, 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 ($P < 0·01$ and $P < 0·05$, 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 ($P = 0·00055$ 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).

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	16·3 ± 11·3	15·4 ± 10·8	15·3 ± 10·8	16·5 ± 11·2	NS	NS	NS	NS
To the first visit at clinic (hours)								
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.
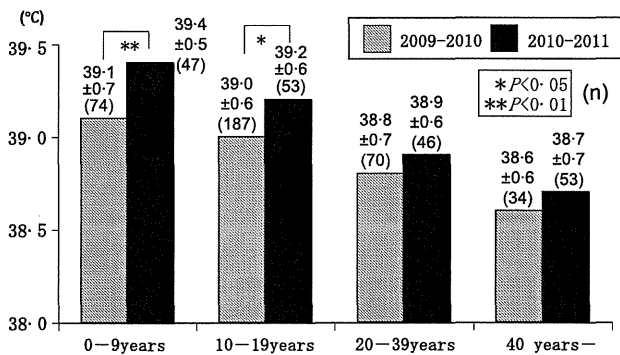
Figure 1. The peak body temperature (°C) of patients with A(H1N1)pdm09 in the 2009–2010 and 2010–2011 seasons, by age. The peak body temperature was significantly higher in the 2010–2011 than the 2009–2010 seasons in the 0–9 and 10–19 years' age groups.

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

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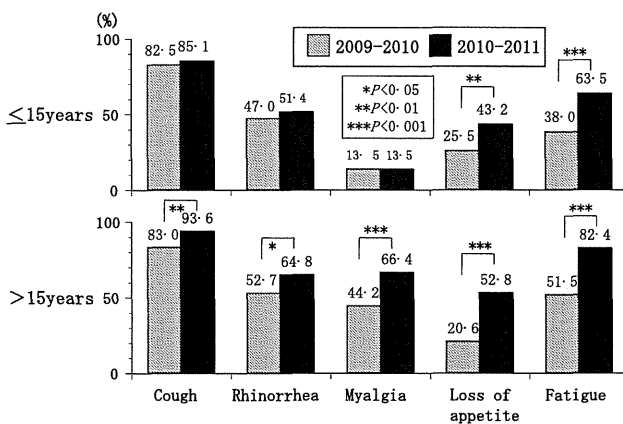
Figure 2. The percentages of the symptoms suffered by patients with A(H1N1) pdm09 infection, by season. The percentage of patients with loss of appetite or fatigue was significantly higher in the 2010–2011 season than in the previous season in children 15 year or younger. The percentage of patients with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

| Duration of fever after the first dose, hour | 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)
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.

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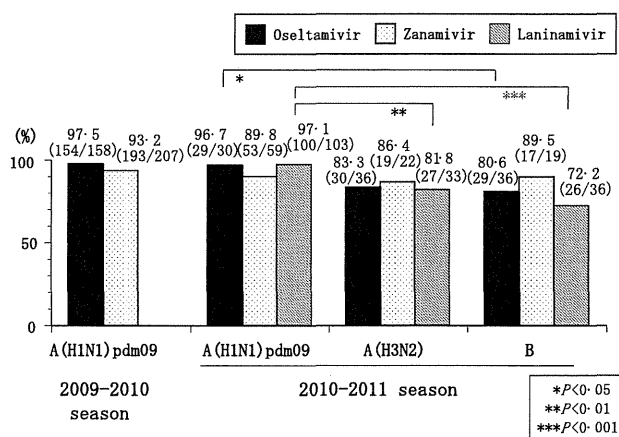
Figure 3. The percentage of patients afebrile at 48 hours after the first dose of each neuraminidase inhibitor. The percentage of patients afebrile at 48 hours after the first dose was significantly higher for A(H1N1)pdm09 than for A(H3N2) (laninamivir) or B (oseltamivir and laninamivir). No significant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

vir 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.

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 $\mu g/kg$ 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.

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ORIGINAL ARTICLE

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

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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.

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

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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/real timeptpcr/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)
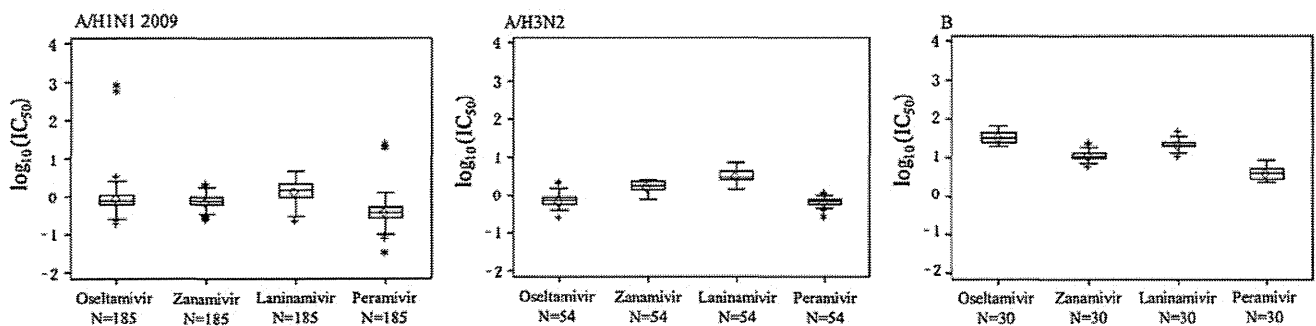
Fig. 1 Half maximal inhibitory concentration (IC_{50}) quartiles of each neuraminidase inhibitor (NAI) for different influenza types. *Diamond* arithmetic mean, *plus symbol* values between 1.5 × IQR and narrow range, except for two viral isolates of A(H1N1)pdm09. The two A(H1N1)pdm09 isolates showed 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).

3 × IQR from UQ/LQ; *asterisk* values above/below 3 × IQR from UQ/LQ, respectively. *IQR* interquartile range, *UQ* 75 percentile, *LQ* 25 percentile

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
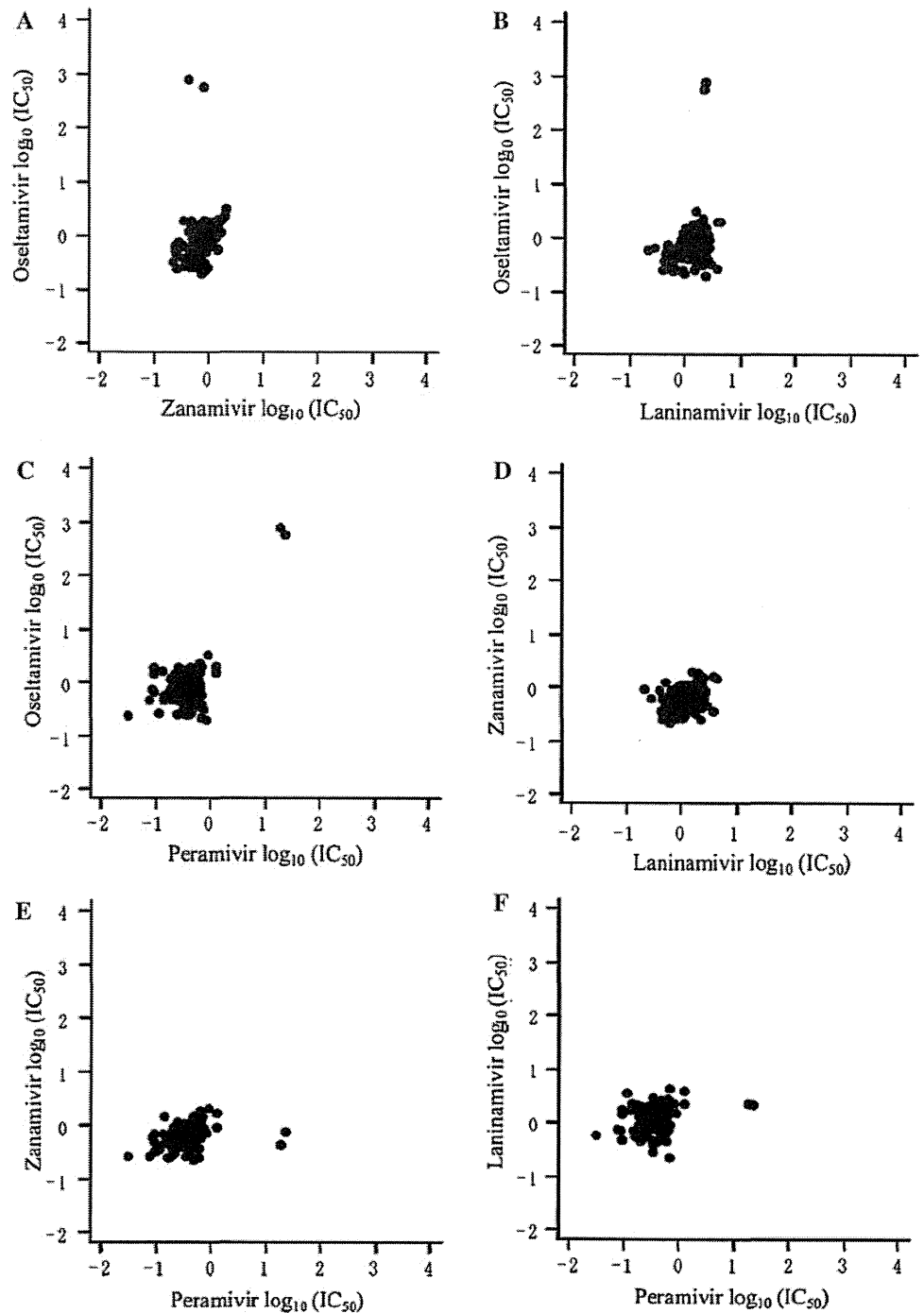
IC_{50} for B to that of H3N2 was especially high in oseltamivir compared with the other three NAIs. It has been reported that the clinical effectiveness of oseltamivir is inferior to influenza B in comparison with influenza A [2]. The clinical efficacy of each drug has not been evaluated in this study. 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.

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ORIGINAL ARTICLE

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

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Abstract Comparison of the viral persistence of pandemic H1N1 (H1N1pdm) and seasonal H1N1 with or without H275Y mutation after oseltamivir therapy has not been adequately done. Virus was isolated before and on days 4–6 from the start of oseltamivir treatment for 158 cases of seasonal (2007–2008 and 2008–2009 seasons) or pandemic (2009–2010 season) H1N1 influenza. Sequence analysis was done for each season and NA inhibition assay (IC_{50}) was done in the 2009–2010 season. H275Y mutation before therapy was 0% in the 2007–2008 and 2009–2010 seasons, but 100% in the 2008–2009 season. Fever and other symptoms were noticeably prolonged after oseltamivir therapy for children with H275Y mutated seasonal H1N1 (2008–2009 season), but not in patients with seasonal H1N1 without mutation (2007–2008) or H1N1pdm (2009–2010). The viral persistence rate was significantly higher for patients 15 years or younger than for those 16 years and older with H275Y mutated seasonal H1N1 (46.2% and 10.5%, respectively) or with H1N1pdm (43.3% and 11.5%, respectively). The H275Y mutation emerged

after oseltamivir treatment in 2.4% (2/82) of all patients with H1N1pdm. In two children, the H275Y mutation emerged after therapy and the IC_{50} increased more than 200 fold; however, the prolongation of fever was not so prominent. In conclusion, oseltamivir was effective for fever and other clinical symptoms; however, the virus persisted longer than expected after treatment in H1N1pdm influenza-infected children in the 2009–2010 season, similar to seasonal H1N1 with H275Y mutation in the 2008–2009 season.

Keywords Influenza A(H1N1) · Oseltamivir ·
Viral shedding · H275Y mutation · IC_{50}

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Introduction

The prodrug oseltamivir phosphate (oseltamivir), an oral neuraminidase (NA) inhibitor, had been effective against seasonal influenza A infection until the 2007–2008 season [1–7], but became less effective in the 2008–2009 season, when seasonal H1N1 influenza with the H275Y mutation [8, 9] was widespread throughout Japan [10, 11]. The reduction was more prominent in children than in adults [10, 11], however, oseltamivir was effective for the pandemic H1N1 (H1N1pdm) influenza that emerged in 2009, as shown by our previous study estimating the duration of fever after the start of oseltamivir therapy [12].

There is great concern about the length of the viral shedding period after oseltamivir therapy for H1N1pdm infection, because longer viral shedding holds the possibly of inducing secondary infection in the home or community. A longer viral shedding period may also be related to the emergence of viral mutation of H1N1pdm viruses [7, 10, 13]. Although the frequency of H275Y mutation of

H1N1pdm virus was reported to be very low in the 2009–2010 season [13, 14], the rapid emergence of oseltamivir-resistant H1N1pdm virus during oseltamivir therapy has been recently reported in hospitalized patients [15, 16]. Viral persistence and the emergence of H275Y mutation during oseltamivir therapy for H1N1pdm has not been adequately analyzed in outpatients.

In this study, we investigated the persistence of symptoms and viruses and the emergence of H275Y NA mutation after oseltamivir therapy for Japanese H1N1pdm patients in a comparison with seasonal H1N1 with or without H275Y mutation. IC_{50} values were also calculated for H1N1pdm before and after therapy.

Methods

Patients

Patients with influenza-like illnesses with findings such as body temperature $\geq 37.5°C$, upper respiratory tract symptoms, and systemic symptoms were tested with antigen detection kits to confirm the presence of influenza A or B in the 2007–2008, 2008–2009 and 2009–2010 seasons. Family doctors, pediatricians, and physicians at 8 clinics (1 clinic each in Gifu, Kumamoto, Gunma, Kanagawa, and Tokushima Prefectures and 3 clinics in Ishikawa Prefecture) in the 2007–2008 and 2008–2009 seasons and at 11 clinics (1 clinic each in Gifu, Kumamoto, Gunma, Kanagawa, and Tokushima Prefectures and 3 clinics each in Ishikawa and Fukuoka Prefectures) in the 2009–2010 season participated in the study. We enrolled, in this study, consecutively, 204 patients (2007–2008, 59 patients; 2008–2009, 54 patients; 2009–2010, 91 patients) with influenza A diagnosed by commercial antigen detection kits who received oseltamivir treatment within 48 h of symptom onset after obtaining informed consent; 170 of 204 patients (47 in 2007–2008; 34 in 2008–2009; 89 in 2009–2010) had influenza A(H1N1) infection confirmed by hemagglutinin inhibition (HAI) test; 12 patients who did not visit the clinic after oseltamivir therapy were excluded from the study, leaving the data of 158 (44 in 2007–2008; 32 in 2008–2009; 82 in 2009–2010) available for analysis. None of the patients had complications from other diseases.

Oseltamivir (adults and children weighing ≥ 37.5 kg: 75 mg; children weighing <37.5 kg: 2 mg/kg) was administered orally, twice a day, for 5 days to all patients. Oseltamivir has been reported to be related to the neuropsychiatric symptoms of young adults and has been prohibited, in most cases, for use by patients aged from 10 to 19 years in Japan. A warning letter concerning the neuropsychiatric symptoms possibly induced by oseltamivir in young adults appeared on the following website (in Japanese): http://www.mhlw.go.jp/houdou/2007/03/h0320-1.html. Therefore, the decision to administer oseltamivir was left to the discretion of the clinician, who followed the foregoing guidelines and patient preference. Patients took the initial dose of oseltamivir at a clinic or at home immediately after the diagnosis of influenza by a commercial antigen detection kit. Antipyretics were not administered, except for acetaminophen, which was used temporally in a few cases.

Age, sex, vaccination status, antigen detection kit test result, and date and time of fever onset were recorded at the first clinic visit. Patients or family members were asked to measure the patient's body temperature at 8:00 a.m. and 8:00 p.m. each day. Body temperature before treatment or at either 8:00 a.m. or 8:00 p.m., whichever was highest, on days 2, 3, and 4 after the start of oseltamivir treatment was analyzed. Patients or family members were also asked to record, at 8:00 a.m. and 8:00 p.m. each day, a symptomatic score (score 0, none; score 1, mild; score 2, moderate; score 3, severe) for six clinical symptoms: nasal symptoms (rhinorrhea or nasal obstruction), cough, sore throat, myalgia or joint pain, general fatigue, and headache.

Antigen detection test kits and virus isolation

Commercial antigen detection kits based on immunochromatography [Capilia FluA+B (Alfresa Pharma), QuickNavi-Flu (Denka Seiken), QuickVue Rapid-SP influ (DS Pharma Biomedical), and Imuno Ace Flu (Touns)] were mainly used.

Viruses were isolated before oseltamivir treatment and on days 4–6 after the start of treatment [7]. We calculated the persistence rate as the ratio of the number of patients in whom virus was detected on days 4–6 after the start of oseltamivir treatment to the number of patients for whom the virus was detected before treatment. Nasopharyngeal swabs were collected from the patient at the first and the second visits, on days 4–6 after the start of treatment. The swabs were placed in viral transport medium (Microtest, Multi-Microbe Media, USA). Viral isolation was done by the standard method using Madin–Darby canine kidney (MDCK) cells (DS Pharma Biomedical, Osaka, Japan). The influenza A(H1N1) subtype of the isolated viruses were determined by HAI test with serum HAI antibodies (Denka Seiken, Tokyo, Japan). The virus isolation and HAI test were performed by Mitsubishi Chemical Medience, Tokyo, Japan.

NA inhibition assay

Viral sensitivity to inhibition by oseltamivir carboxylate (OC) (F. Hoffmann-La Roche, Basel, Swiss Confederation) was determined by phenotyping, using a NA-Star

chemiluminescent substrate-based NA enzyme assay. This phenotyping assay has been well established and is widely used as part of ongoing global influenza surveillance programs [17, 18]. A detailed description of the assay principles and performance can be found on the website of the Neuraminidase Inhibitor Susceptibility Network (NISN): http://www.nisn.org/v_ic50_methodology.html or applied biosystems: http://www.appliedbiosystems.jp/website/CONTENTS/NA-Star_protocol.pdf. The phenotyping assay was performed by ViroClinic, Rotterdam, The Netherlands.

NA sequence analysis

MDCK culture aliquots were shipped to RIKEN Omics Science Center (RIKEN Yokohama Institute, Japan) where reverse transcription-polymerase chain reaction (RT-PCR) and sequencing of the NA gene [19] were done. Viral RNA was successfully amplified from the baseline sample, and the rgw NA sequence was consistent with pandemic influenza A(H1N1). Extracted RNA was transcribed into cDNA by multi-segment RT-PCR with 5'-ACGCGTG ATCAGCAAAAGCAGG-3' and 5'-ACGCGTGATCAG TAGAAAGG-3' [19]. For sequencing of the pandemic 2009 N1NA gene, corresponding cDNAs were amplified by PCR using 5'-ACGCGTGATCAGCAAAAGCAGG-3' (forward) and 5'-ATTAGGGTTCGATATGGGCT-3' (reverse) primers with the first cDNA fragment, 5'-CC TTGGAATGCAGAACCTTC-3' (forward) and 5'-GATT GTCTCCGAAAATCCCA-3' (reverse) primers with the second fragment, 5'-AAAGGGAAAGATAGTCAAAT-3' (forward), and 5'-ACGCGTGATCAGTAGAAACAAGG-3' (reverse) primers with the third fragment.

Statistical analysis

The Mann–Whitney U test was used for between-group comparisons of median values concerning age, body temperature, total symptom score, IC_{50}, time from onset of symptoms to sampling, and the interval between the first and second virus sampling. Fisher's exact test was also done to compare between group percentages of the persistence rates of virus, male-to-female ratio, and vaccination status. $P < 0.05$ was considered statistically significant.

Results

Patient characteristics and H275Y mutation before therapy

Of 158 patients with influenza A(H1N1) virus infection, 44 presented during the 2007–2008 season (December 1,

2007–February 27, 2008), 32 during the 2008–2009 season (December 1, 2008–April 30, 2009), and 82 during the 2009–2010 season (November 1, 2009–April 30, 2010). No H275Y mutation was detected before therapy by NA sequence analysis in seasonal H1N1 in 2007–2008 or in H1N1pdm in 2009–2010, but in all seasonal H1N1 in 2008–2009. Patient demographic characteristics for seasonal H1N1 without H275Y mutation (2007–2008), seasonal H1N1 with H275Y mutation (2008–2009), and H1N1pdm (2009–2010) are summarized in Table 1. No significant pretreatment differences among the groups were found for median values of age, body temperature, or total symptom score, male-to-female ratio, or vaccination status. The median (25th–75th percentile) time from onset of symptoms to sampling was 13.8 (7.1–22.1) h in the 2007–2008, 19.7 (12.8–29.9) h in the 2008–2009, and 19.8 (14.0–26.9) h in the 2009–2010 seasons (2007–2008 vs. 2008–2009, $P = 0.071$; 2007–2008 vs. 2009–2010, $P = 0.010$; 2008–2009 vs. 2009–2010, $P = 0.962$).

Body temperature before and after the start of therapy

Figure 1 shows the mean value of the highest body temperature on day 1 (before therapy), and days 2, 3, and 4 after starting oseltamivir therapy for seasonal H1N1 with or without H275Y mutation and for H1N1pdm.

For adults 16 years and over, the mean values of fever of all three groups declined to less than 37°C on day 3 or 4 after starting oseltamivir therapy. For children 15 years and under, the mean value of fever declined to less than 37°C on day 3 or 4 in seasonal H1N1 without H275Y mutation, but remained greater than 37°C on day 3 or 4 in seasonal H1N1 with the H275Y mutation. In H1N1pdm, the mean value of fever declined to under 37°C on day 3 or 4 in children, similar to seasonal H1N1 without H275Y mutation.

Persistence of other symptoms after therapy

The persistence rate of symptoms was calculated as the number of patients with each symptom at the second virus sampling on days 4–6 after the start of therapy divided by the number of patients in each patient group.

The persistence rates of the six symptoms for seasonal H1N1 without (2007–2008) or with (2008–2009) H275Y mutation and H1N1pdm (2009–2010) were 7.1% (1/14), 61.5% (8/13), and 30% (9/30), respectively ($P = 0.004$ between 2007–2008 and 2008–2009), for children 15 years and younger. The rates for adults 16 years and older were 36.7% (11/30), 42.1% (8/19), and 32.7% (17/52), respectively, with no significant differences among the three groups.

Table 1 Baseline demographic characteristics of patients with seasonal or pandemic H1N1 influenza

	Seasonal H1N1		H1N1pdm (2009–2010)
	H275Y mutation (–) (2007–2008)	H275Y mutation (+) (2008–2009)	
n	44	32	82
Age (years)[a]	33.5 (7–41.3)	24.5 (5.5–31.8)	25.5 (8.3–39.8)
Male/female	27/17	14/18	37/45
Vaccination[b] (positive/negative/unknown)	10/34/0	11/21/0	26/54/2
BT before therapy (°C)[a]	38.3 (37.7–38.8)	38.0 (37.4–38.8)	38.2 (37.8–38.7)
Total symptom score[a,c]	8 (6–11)	8 (5–10)	7 (4–10)

No significant difference was found in any of the parameters for the 2007–2008, 2008–2009, and 2009–2010 seasons

BT body temperature

[a] Median (25–75 percentile)

[b] Vaccination, vaccination for seasonal influenza

[c] Total symptom score, total of the individual scores for the following six symptoms: nasal symptoms, cough, sore throat, myalgia or joint pain, general fatigue, and headache (score 0, none; score 1, mild; score 2, moderate; score 3, severe)

Fig. 1 Mean body temperature before, and on days 2, 3, and 4 after, the start of therapy in the 2007–2008, 2008–2009, and 2009–2010 seasons. Mean body temperature above 37.0°C was seen not only before day 2, but also on day 3 or 4 (*numbers enclosed in boxes*) in children in the 2008–2009 season in which H1N1 with the H275Y mutation prevailed
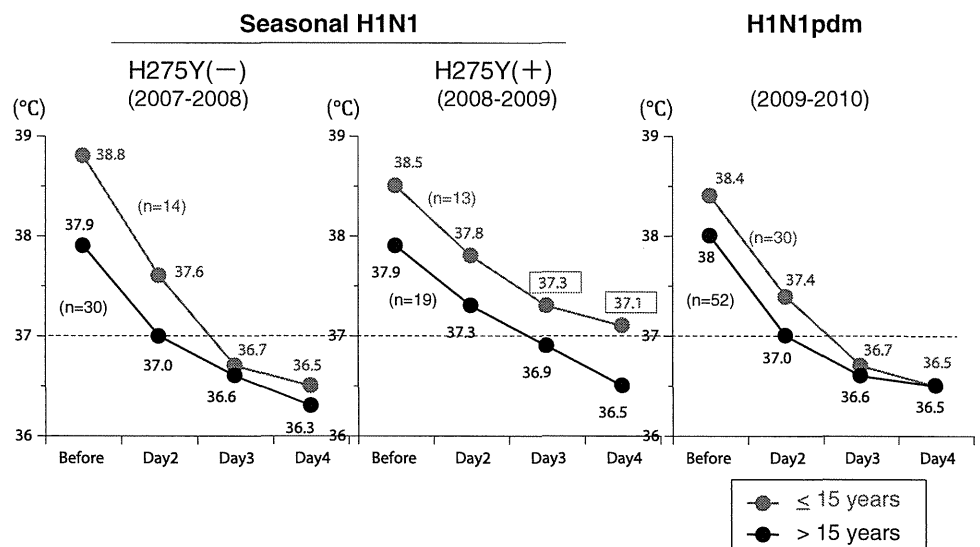
The persistence rates for cough were 7.1% (1/14), 46.2% (6/13), and 16.7% (5/30), respectively ($P = 0.033$ between 2007–2008 and 2008–2009), in children. The rates for adults were 20.0% (6/30), 42.1% (8/19), and 23.1% (12/52), respectively (NS among the three groups).

The persistence rates for the nasal symptoms of children were 7.1% (1/14), 61.5% (8/13), and 13.3% (4/30), respectively ($P = 0.004$ between 2007–2008 and 2008–2009, $P = 0.003$ between 2008–2009 and 2009–2010). The rates for adults were 10% (3/30), 21.1% (4/19), and 9.6% (5/52) in each season (NS among the three groups).

The persistence rates for sore throat (0–21.1%), myalgia or joint pain (0–6.7%), general fatigue (0–9.6%), and headache (0–3.3%) were low and without significance among the three groups for both children and adults.

Virus persistence after oseltamivir therapy

The interval between the first and second virus sampling was significantly longer in the 2008–2009 and 2009–2010 seasons (median of 5 days and 25th–75th percentile of 4–5 days in both seasons) than in the 2007–2008 season (median of 4 days and 25th–75th percentile of 4–5 days; $P = 0.014$ and $P = 0.002$, respectively), even though the study protocol was unchanged throughout the three seasons. No significant differences were found in the persistence rates of A(H1N1) virus after oseltamivir therapy among the three groups of adults 16 years and older (2007–2008, 10%; 2008–2009, 10.5%; and 2009–2010, 11.5%) (Fig. 2). In children 15 years and younger, there was also no statistically significant difference in the rates
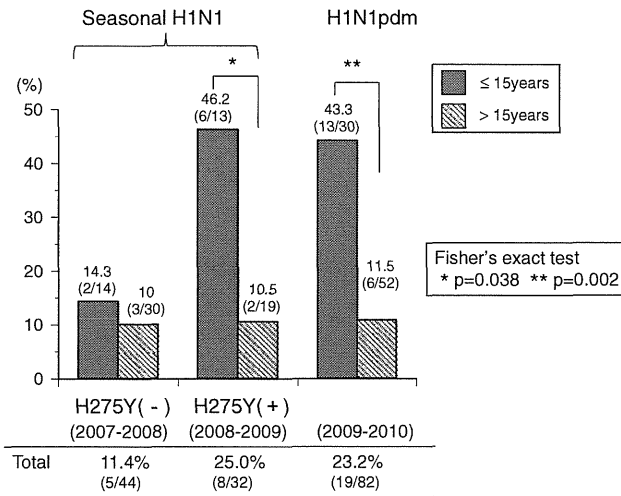
Fig. 2 Persistence rate of virus on the 4th–6th days after the start of therapy. The rate was significantly higher for children ≤15 years (*solid bars*) than for adults 15 years and older (*hatched bars*) in both 2008–2009 and 2009–2010 seasons

Table 2 H1N1pdm virus persistence rates in the 2009–2010 season by age cohort

Age	Persistence rates
0—5 years	71.4% (5/7)
6—10 years	35.0% (7/20)
11—15 years	33.3% (1/3)
16 years—	11.5% (6/52)

Fisher's exact test
* p=0.002 ** p=0.037

Fisher's exact test: * $P = 0.002$; ** $P = 0.037$

for the three seasons; however, the rate was higher in 2009–2010 and 2008–2009 than in 2007–2008 (43.3%, 46.2%, and 14.3%, respectively). The rates were significantly higher for children than for adults in both the 2008–2009 ($P = 0.038$) and 2009–2010 ($P = 0.002$) seasons (Fig. 2).

The persistence rates of A(H1N1) virus on day 4, day 5, and day 6 in all ages were 10.3%, 16.7%, and 0.0% in the 2007–2008 season, 33.3%, 26.7%, and 0.0% in the 2008–2009 season, and 34.5%, 14.6%, and 25.0% in the 2009–2010 season. No significant differences of the persistent rates were shown among days 4, 5, and 6 in each season.

For H1N1pdm in the 2009–2010 season, the viral persistence rate was significantly higher for patients aged 0–5 years (71.4%) than for those aged 16 years or older (11.5%; $P = 0.002$). It was also higher for patients aged 6–10 years (35.0%) than for patients 16 years or older (11.5%; $P = 0.037$) (Table 2).

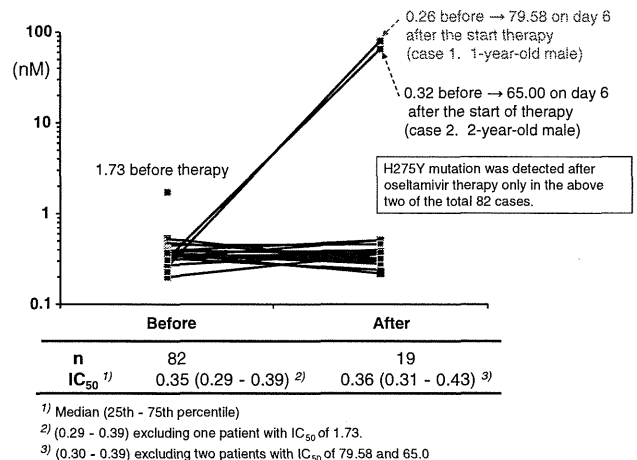### H275Y mutation after therapy and IC50

By NA sequence analysis, H275Y mutation was shown to have emerged after oseltamivir therapy in only two children with H1N1pdm in the 2009–2010 season. The frequency of emergence of H275Y mutation after oseltamivir therapy was 2.4% (2/82) for all patients and 6.7% (2/30) for children 15 years and younger. The frequency of patients in whom the virus persisted after oseltamivir therapy was 10.5% (2/19) of all patients and 15.4% (2/13) of children.

Fig. 3 IC$_{50}$ for oseltamivir before and on days 4–6 after the start of therapy for patients with pandemic H1N1 in the 2009–2010 season. IC$_{50}$ for oseltamivir was increased approximately 200- to 300 fold in two patients in whom the H275Y mutation emerged

The median IC$_{50}$ was 0.35 nM (25th–75th percentile of 0.29–0.39 nM) before therapy and 0.36 nM (25th–75th percentile of 0.31–0.43 nM) after therapy (Fig. 3). The IC$_{50}$ was increased 306 fold, from 0.26 to 79.58 nM (case 1, 2-year-old boy), and 203 fold, from 0.32 to 65.0 nM (case 2, 1-year-old boy) in two patients on day 6 after the start of oseltamivir therapy (Fig. 3). In both cases, H275Y mutation emerged after oseltamivir therapy. The highest body temperature of each day for case 1 was 38.3°C on day 1, 38.9°C on day 2, 37.6°C on day 3, 36.7°C on day 4, and 37.4°C on day 5; for case 2, highest body temperatures were 38.7°C on day 1, 36.6°C on day 2, 36.5°C on day 3, 36.6°C on day 4, and 36.6°C on day 5.

Discussion

Higher mortality rates (deaths per million population) by H1N1pdm 2009 were reported in many countries (Canada, 2.8; UK, 2.2; Mexico, 2.9; USA, 3.3; South Africa, 1.8; Argentina, 14.6; Australia, 8.6; Brazil, 7.0; Chile, 8.1; and New Zealand, 4.4) than in Japan, where the rate was extremely low (0.2) [20]. The wide use of commercial antigen detection kits by skilled physicians and the early start of anti-influenza drug therapy in Japan probably contributed to these results.

We previously reported in clinical and virological studies that oseltamivir was effective against seasonal influenza A(H3N2) and A(H1N1) until the 2007–2008 season, but that it was less effective for seasonal H1N1 with the H275Y mutation, especially in children [5–7, 10, 11]. In this study, no H275Y mutation was detected before treatment of H1N1pdm, and oseltamivir seemed to be effective for H1N1pdm in the 2009–2010 season, similar to seasonal H1N1 without the H275Y mutation (2007–2008 season) in terms of the rapid decline of fever and disappearance of other symptoms. However, viral persistence evaluated by virus culture was long for H1N1pdm, similar to seasonal H1N1 with H275Y mutation in the 2008–2009 season, especially in children 15 years and younger [10, 11]. We analyzed the viral persistence of patient cohorts 0–5, 6–10, 11–15, and 16 years of age and older in the 2009–2010 season, and the rate decreased with age.

In the 2008–2009 season, viral persistence was long because of reduced effectiveness of oseltamivir to the H275Y mutated virus [10]. However, the sensitivity of the virus to oseltamivir in the 2009–2010 season as evaluated by IC_{50} was quite comparable to that of seasonal H1N1 without H275Y mutation [10]. A long virus shedding period has also been reported, by RT-PCR, for young H1N1pdm patients [21–23]. The reason for the long virus persistence, irrespective of low IC_{50} of oseltamivir to H1N1pdm, is not clear. One possible explanation is that the long virus shedding period in H1N1pdm without H275Y mutation may be related to a low level of acquired immunity to a newly emergent influenza virus. Exposure to the seasonal H1N1 virus, which has similar immunological characteristics to H1N1pdm, may give some protection to the infected patients through cross-reactivity [24]. The low prevalence of H1N1pdm for persons more than 50 years old [25] and the excellent elevation of antibody titer by a single vaccination for H1N1pdm [26] in the 2009–2010 season seem to support this hypothesis. It should be noted that seasonal H1N1 virus cleared relatively early, even in children less than 16 years of age treated with oseltamivir. The long virus shedding after treatment with oseltamivir in young patients may be a characteristic of the H1N1pdm virus.

For H1N1pdm, the pre-therapy rate of H275Y mutation was low in this study (0%) similar to the other reports of the 2009–2010 season [13, 14]; however, the rate of this mutation after oseltamivir therapy has not been clearly studied, especially in outpatient clinics. In this study, H275Y mutation and 200- to 300-fold increases of IC_{50} were found in two children (2.4% of all subjects; 6.7% of children) after oseltamivir therapy. The H275Y mutation in our study may have been selected under oseltamivir pressure. The two patients did not show an especially prominent prolongation of fever, until day 4, and were cured without complication. No emergence of H275Y mutation after therapy was found for the adult outpatients of this study, and no E119V or N295S mutation reported to be related to oseltamivir resistance was detected [27]. However, it is important to pay careful attention to the appearance of H275Y mutation during or after either oseltamivir or peramivir therapy for patients with H1N1pdm in addition to the community-acquired H275Y mutation detected before therapy [16, 28].

In conclusion, oseltamivir was effective for fever and other clinical symptoms; however, viral persistence was longer than expected in children with H1N1pdm influenza in the 2009–2010 season. The frequency of H275Y mutation of H1N1pdm was low (2.4%) in this study of outpatients undergoing oseltamivir therapy.

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肺炎球菌ワクチンの新しい展開

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