the NA inhibitors oseltamivir or zanamivir for pandemic H1N1 2009 virus infection have been done [5–7]; however, comparative studies with seasonal H1N1 virus infection have not been adequately reported.

In this report, we compare the clinical symptoms and the duration of fever ≥37.5°C after the first dose of oseltamivir or zanamivir and after the onset among patients with seasonal H1N1 in the 2007–2008 and 2008–2009 seasons and with pandemic H1N1 2009 virus infection.

Methods

Study procedures

Family doctors, pediatricians, and physicians at 15 clinics that belong to the Influenza Study Group of the Japan Physicians Association participated in the study. Patients were enrolled from December 7, 2007 through March 27, 2008 in the 2007–2008 season, from December 6, 2008 through February 14, 2009 in the 2008–2009 season, and from August 11, 2009 through January 31, 2010 in the 2009–2010 season. Patients who reported to any of our 15 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 a commercial antigen detection kit. From all outpatients with influenza, diagnosed by antigen detection kit and/or clinical symptoms and without severe underlying diseases such as chronic obstructive pulmonary disease or chronic heart disease, those who received oseltamivir or zanamivir within 48 h after the onset of symptoms were registered in this study after providing informed consent. Excluded from the analysis were four patients in serious condition who were sent immediately to a hospital.

Oseltamivir has been reported to be related to neuropsychiatric symptoms of young adults and has been prohibited, in most cases, for use by patients aged from 10 to 19 years in Japan. Zanamivir is not recommended for patients with underlying respiratory disease or children under 5 years. Therefore, the decision on whether to administer oseltamivir or zanamivir to patients with influenza was left to the discretion of the patient's physician, who followed the above guidelines and patient preference.

Specimens from throat swabs, nasal 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, Capilia FluA+B (Alfresa Pharma), QuickVue Rapid-SP influ (DS Pharma Biomedical), QuickNavi-Flu (Denka Seiken), and Imuno Ace Flu (Touns), were mainly used.

Viral isolation was done with informed consent by standard methods using Madin–Darby canine kidney (MDCK) [8]. The type and subtype of the isolated influenza was determined by the reverse transcriptional polymerase chain reaction (RT-PCR) method using specific primer sets for seasonal influenza as described elsewhere [8]. New primers for AH1N1 pandemic 2009 were synthesized, and their sequences were as follows: a forward external primer, 5′-GTG CTA TAA ACA CCA GCC TC-3′ (NA nucleotide position 902–922); a forward external primer, 5′-GCC ACA GGA TTG AGG AAT GT-3′ (NA nucleotide position 994–1013); and a reverse primer 5′-CCT GCT CAT TTT GAT GGT GA-3′ (NA nucleotide position 1123–1104).The subtype of influenza H1N1 was determined by the RT-PCR method using subtype-specific primer sets for A/Mexico/4603/2009(H1N1) HA gene, 5′-GTG CTA TAA ACA CCA GCC TC-3′ (forward 902–922), 5′-GCC ACA GGA TTG AGG AAT GT-3′ (insert 994–1013), and 5′-CCT GCT CAT TTT GAT GGT GA-3′ (reverse 1123–1104).

A neuraminidase gene segment was amplified by RT-PCR, and the presence of the H275Y mutation was determined by nucleotide sequencing for 166 patients with H1N1 virus: 44 consecutive patients in the 2007–2008 season, 88 in the 2008–2009 season, and 34 in the 2009–2010 season, including 77 males and 89 females of mean age 26.6 ± 18.5 years.

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 5 days. Zanamivir (10 mg for adults and for children aged 5 years or over) was inhaled twice per day for 5 days. Antipyretics were not 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 oseltamivir or zanamivir, and the resolution of fever were recorded by the physician, patient, or an attending family member. The first time that 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 <37.5°C was attained and maintained for more than 24 h was defined as the time when 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, fatigue, rhinorrhea, sore throat, myalgia, headache, loss of appetite, vomiting, and diarrhea.

Table 1 Baseline demographic characteristics and clinical symptoms of patients with seasonal or pandemic A(H1N1) virus infection

	Seasonal A(H1N1)		2009 Pandemic A(H1N1) (c)	*P* value between		
	2007–2008 (a)	2008–2009 (b)		(a) and (b)	(b) and (c)	(a) and (c)
Number of patients	68	193	361			
Age, mean years ± SD (range)	26.1 ± 20.2 (1–69)	22.0 ± 18.0 (9 months–90)	18.4 ± 13.2 (1–78)	NS	<0.05	<0.01
Male/female	39/29	101/92	180/181	NS	NS	NS
Vaccination[a] (positive/negative/unknown)	28/40/0	80/112/1	73/284/4	NS	<0.001	<0.001
Peak body temperature (°C)	39.0 ± 0.8	39.0 ± 0.6	39.0 ± 0.7	NS	NS	NS

[a] Vaccination for seasonal influenza

All data were collected using an Internet-based protocol based on a server located in a secure room at the Gifu City Medical Association [9]. The time from the initial administration of oseltamivir or zanamivir to the resolution of fever and the duration of fever between the onset and resolution were calculated automatically in the SQL database [10, 11]. All study-related documents and procedures were approved by the institutional review board at Hara-Doi Hospital.

Statistical analysis

Student's *t* test was used for between-group comparisons of the duration of fever. The Fisher exact test was also used to compare between-group differences in the percentage of patients. A *P* value <0.05 was considered statistically significant.

Results

Patient characteristics

A total of 733 patients were enrolled in the three seasons studied. The complete data of 685 influenza patients were available for analysis: 68 H1N1 patients aged 1–69 years in the 2007–2008 season, 193 H1N1 patients aged 9 months–90 years in the 2008–2009 season, and 361 pandemic H1N1 patients aged 1–78 years in the 2009–2010 season. The demographic characteristics of the patients are summarized in Table 1.

The mean age and the percentage of patients vaccinated for seasonal influenza were significantly lower in the pandemic season than in the seasonal H1N1 seasons. The mean peak body temperature was the same (39.0°C) for all three seasons. All 68 patients were positive by commercial antigen detection kit for influenza in 2007–2008, as were all 193 in 2008–2009 and 342 in the 2009–2010: a negative reaction with commercial antigen detection kit was found for 19 patients in the 2009–2010 season.

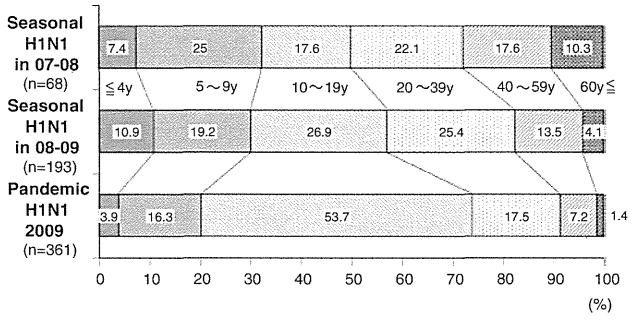

Fig. 1 Age distribution of patients with seasonal H1N1 in the 2007–2008, 2008–2009, and pandemic H1N1 2009 seasons. The percentage of patients aged 10–19 years was significantly higher for pandemic H1N1 than for seasonal H1N1 in the 2007–2008 and 2008–2009 seasons

The percentage of patients aged 10–19 years was significantly higher in the 2009 pandemic season (53.7%) than in the 2007–2008 (17.6%) and 2008–2009 (26.9%) H1N1 seasons ($P < 0.001$, Fig. 1).

Clinical symptoms

The symptoms of the patients at the start of NAI therapy are shown in Table 2. The percentage of patients with fatigue, rhinorrhea, sore throat, myalgia, headache, and loss of appetite was significantly lower for pandemic 2009 than for seasonal H1N1 in 2008–2009. The percentage with fatigue was also significantly lower for pandemic 2009 than for seasonal H1N1 in 2007–2008 ($P < 0.01$). No significant differences were found in the percentage of patients with body temperature >37.4° or 37.9°C, cough, vomiting, and diarrhea among the three seasons.

Duration of fever after administration of the first dose of an antiinfluenza drug and after the onset

Of 68 patients with influenza H1N1 in the 2007–2008 season, 41 were treated with oseltamivir and 27 with zanamivir. In the 2008–2009 season, 87 patients with H1N1 were treated with oseltamivir and 106 were treated with zanamivir, and

Table 2 Clinical symptoms of patients with seasonal or pandemic H1N1 at the start of antiinfluenza drug therapy

	Seasonal A(H1N1)		2009 Pandemic	*P* value between		
	2007–2008 (a)	2008–2009 (b)	A(H1N1) (c)	(a) and (b)	(b) and (c)	(a) and (c)
Clinical symptoms at the first visit (%)						
Body temperature ≥37.5°C	95.6	95.9	97.0	NS	NS	NS
Body temperature ≥38.0°C	77.9	75.1	76.2	NS	NS	NS
Cough	85.3	81.3	78.9	NS	NS	NS
Fatigue	63.2	62.2	41.3	NS	<0.001	<0.01
Rhinorrhea	57.4	71.0	47.4	NS	<0.001	NS
Sore throat	42.6	50.3	32.7	NS	<0.001	NS
Myalgia	35.3	37.3	26.3	NS	<0.01	NS
Headache	30.9	51.8	30.5	<0.01	<0.001	NS
Loss of appetite	29.4	34.2	20.8	NS	<0.001	NS
Vomiting	4.4	3.1	3.6	NS	NS	NS
Diarrhea	1.5	4.7	2.8	NS	NS	NS

Table 3 Patient characteristics by treatment

	Seasonal A(H1N1)		2009 Pandemic
	2007–2008	2008–2009	A(H1N1)
Oseltamivir therapy group			
Number of patients	41	87	149
Age, mean years ± SD	26.6 ± 22.0	22.4 ± 21.6	21.6 ± 17.8
Male/female	24/17	47/40	72/77
Vaccination[a] (positive/negative/unknown)	16/25/0	36/50/1	40/109/0
Peak body temperature, °C	39.0 ± 0.7	39.0 ± 0.6	38.9 ± 0.7
Time to the first administration of the drug after the onset, mean hours ± SD	18.1 ± 10.6	17.3 ± 11.6	17.5 ± 11.7
Zanamivir therapy group			
Number of patients	27	106	212
Age, mean years ± SD	25.4 ± 17.0	22.1 ± 14.4	16.1 ± 7.8
Male/female	12/15	54/52	108/104
Vaccination (positive/negative/unknown)	10/17/0	44/62/0	33/175/4
Peak body temperature, °C	39.1 ± 0.8	38.9 ± 0.7	39.0 ± 0.6
Time to the first administration of the drug after the onset, mean hours ± SD	16.5 ± 8.8	15.7 ± 11.1	17.7 ± 10.7

[a] Vaccination for seasonal influenza

149 and 212 patients with pandemic H1N1 were treated with oseltamivir and zanamivir, respectively (Table 3). There were no significant differences in age, male-to-female ratio, vaccination status, peak body temperature or time to the first administration of the drug after the onset between the oseltamivir and zanamivir therapy groups.

Minor adverse reactions were observed for five patients treated with oseltamivir and for eight patients treated with zanamivir. No severe adverse reactions were reported.

The duration of fever after administration of the first dose of oseltamivir or zanamivir for all ages is shown in Table 4. The duration after the start of oseltamivir therapy was significantly shorter for patients with pandemic H1N1 (23.0 ± 11.6 h) than for seasonal H1N1 in the 2008–2009 (49.7 ± 32.3 h) and 2007–2008 seasons (32.0 ± 18.9 h) ($P < 0.001$ and $P < 0.01$, respectively). There was no significant difference in the duration of fever after the start of zanamivir therapy among the three seasons. Significant differences were found between oseltamivir and zanamivir therapy for patients with pandemic 2009 ($P < 0.01$) and seasonal H1N1 in 2008–2009 ($P < 0.001$).

No significant difference was found in the duration of fever after the start of oseltamivir or zanamivir therapy for

Table 4 Duration of fever after the first dose of oseltamivir for pandemic or seasonal A(H1N1) by age (mean hours ± SD)

	After the first dose		*P* between oseltamivir and zanamivir	After the onset		*P* between oseltamivir and zanamivir
	Oseltamivir	Zanamivir		Oseltamivir	Zanamivir	
Seasonal A(H1N1) in the 2007–2008 season	32.0 ± 18.9 ($n = 41$) a	31.5 ± 14.9 ($n = 27$)	NS	50.2 ± 21.4 ($n = 41$) d	48.1 ± 16.9 ($n = 27$)	NS
Seasonal A(H1N1) in the 2008–2009 season	49.7 ± 32.3 ($n = 87$) b	27.3 ± 18.6 ($n = 106$)	<0.001	67.2 ± 33.8 ($n = 87$) e	43.2 ± 20.5 ($n = 106$)	<0.001
2009 Pandemic A(H1N1)	23.0 ± 11.6 ($n = 149$) c	26.9 ± 15.4 ($n = 212$)	<0.01	40.7 ± 15.8 ($n = 149$) f	44.7 ± 16.9 ($n = 212$)	<0.05

a versus b, b versus c: $P < 0.001$

a versus c: $P < 0.01$

d versus e, d versus f: $P < 0.01$

e versus f: $P < 0.001$

pandemic H1N1 between patients ≤15 years of age (oseltamivir 22.5 ± 10.8 h and zanamivir 27.1 ± 16.5 h) and those >15 years of age (23.5 ± 12.5 and 26.6 ± 13.9 h, respectively).

Duration of fever after the onset

The duration of fever after the onset was significantly shorter for patients with pandemic 2009 (40.7 ± 15.8 h) than for patients with H1N1 in both the 2008–2009 (67.2 ± 33.8 h, $P < 0.001$) and 2007–2008 (50.2 ± 21.4 h, $P < 0.01$) seasons (see Table 4). Significant differences were shown between oseltamivir and zanamivir therapy for patients with pandemic in 2009 ($P < 0.05$) and seasonal H1N1 in 2008–2009 ($P < 0.001$).

N1 sequence analysis

Sequence analysis revealed that all 88 of the H1N1 virus isolates in the 2008–2009 season but none in the 2007–2008 or 2009–2010 seasons contained the H275Y mutation.

Discussion

In the 2008–2009 season, almost 100% of the seasonal H1N1 virus in Japan was resistant to oseltamivir because of H275Y mutation [1, 2], but it was reported to be susceptible to amatadine or rimantadine [12]. We reported a lower effectiveness of oseltamivir to the H275Y mutated H1N1 virus in the 2008–2009 season compared to H1N1 virus without H275Y mutation in the 2007–2008 season, especially for children [1, 2]. From August 2009 until February 2010 in Japan, the pandemic H1N1 2009 virus was prevalent, with the seasonal influenza H1N1 and H3N2 viruses rarely found [13]. This pandemic H1N1 2009 influenza virus was genetically different from seasonal H1N1 and had a swine component. It was reported in a genetic and phenotypic analysis to be susceptible to oseltamivir and zanamivir [5]. Therefore, we felt it was important to compare the clinical symptoms of the pandemic H1N1 virus infection and the effectiveness of NAIs against this pandemic virus with seasonal H1N1 virus infection.

The percentage of pandemic 2009 patients with fever (97.0%, >37.4°C) was similar to the report by Dawood et al. [5] (93%). The percentage of patients with sore throat (32.7%) or diarrhea (2.8%) was similar to a report by Cao et al. [6] (36.6% or 2.8%, respectively). The percentages of 2009 pandemic patients with cough (78.9%), fatigue (41.3%), rhinorrhea (47.4%), headache (30.5%), or vomiting (3.6%) of our study were higher than those reported by Cao et al. [6] (69.5%, 10.3%, 23.7%, 19.5%, or 1.9%, respectively).

The clinical symptoms of pandemic and seasonal H1N1 virus infection are said to be similar or rather milder than those of seasonal H1N1 infection, but they have not been adequately compared. In this study, the peak body temperature and the percentage of patients with body temperature ≥37.5° or ≥38.0°C at the start of NAI therapy were equivalent in the three seasons, and the frequency of symptoms was the same or lower for pandemic influenza compared with seasonal H1N1. The pandemic H1N1 2009 infection was a self-limiting illness, as is seasonal influenza, and most patients recovered without complications [5]. However, severe outcomes, including respiratory failure, encephalopathy, myocarditis, and death, have in rare cases been reported. The percentage of patients with intestinal symptoms, such as diarrhea, vomiting, or abdominal pain, was higher for hospitalized or serious patients [5, 7, 14, 15].

The duration of fever after the first dose of a drug and from the onset was analyzed to evaluate the clinical effectiveness of NAIs [10, 11, 16] 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, the duration of fever after the first dose of oseltamivir or zanamivir of patients with influenza A was approximately 30 h [10, 11], with no significant difference in the duration of fever between seasonal H1N1 and H3N2 virus infection [16]. Oseltamivir was less effective for influenza B than for influenza A [10, 16].

The data obtained using these self-recorded reports seem quite adequate and informative.

In this study of the three most recent influenza seasons, zanamivir was equally effective for pandemic and seasonal H1N1. Zanamivir is inhaled as a dry powder and is reported to be deposited at various sites after administration: oropharynx, 77.6%, whole lung, 13.2% (5.1% central lung region, 4.2% intermediate lung lobe, 3.9% peripheral lung region), and trachea, 1.2% [17]. The effectiveness of zanamivir was not different between pandemic and seasonal H1N1, probably because zanamivir acts on the respiratory system directly and is not affected by H275Y mutation.

However, the effectiveness of oseltamivir differed by season. In 2007–2008, oseltamivir had similar effectiveness to zanamivir. In 2008–2009, oseltamivir had reduced effectiveness in comparison with zanamivir and oseltamivir in the previous season, especially for children, as we previously reported [1, 2], because the seasonal H1N1 in the 2008–2009 season had acquired resistance to oseltamivir by H275Y mutation.

No H275Y mutation was detected for our 34 patients with pandemic H1N1 2009. Our data on the duration of fever showed that oseltamivir was more effective for the pandemic influenza than for seasonal H1N1 in both the 2008–2009 and 2007–2008 seasons. Oseltamivir was more effective than zanamivir for 2009 pandemic patients. The reason for the greater effectiveness of oseltamivir for pandemic H1N1 is unclear, and further study is necessary.

In Japan, the administration of zanamivir is not recommended for children under 5 years, and oseltamivir was prohibited for use by patients aged from 10 to 19 years in the 2007–2008 and 2008–2009 seasons. This restriction makes controlled studies of antiinfluenza drugs that include patients at all ages difficult in Japan, for ethical reasons. Although there was no control (untreated) group in this study, comparisons were made between the H1N1 strains from 2007 to 2008, 2008 to 2009, and 2009 to 2010; thus, the lack of traditional controls is mitigated.

The very few patients in serious condition taken immediately to the hospital were excluded from this study. For these serious patients a combination therapy that includes antivirals and other therapies to mitigate complications may be necessary. The mortality rate (death per million population) by pandemic H1N1 2009 was extremely low in Japan (0.2) compared with other countries (Canada, 2.8; UK, 2.2; Mexico, 2.9; US, 3.3; South Africa, 1.8; Argentina, 14.6; Australia, 8.6; Brazil, 7.0; Chile, 8.1; New Zealand, 4.4) [18], probably because the wide use of commercial antigen detection kits in Japan by skilled physicians promotes accurate diagnosis and the early start of antiinfluenza drug therapy, and because universal coverage by healthcare insurance allows for the testing and treatment to be done at a reasonable cost to the patient, which allows more patients to seek timely treatment. Also, the effectiveness of an antiinfluenza drug in shortening the febrile period confirmed in this study seems to have contributed to reductions in the mortality rate and the rate of complications.

It is common for Japanese patients to be examined by commercial antigen detection kits at clinics near their homes and to start taking antiinfluenza drugs immediately after influenza infection is diagnosed. The effectiveness of commercial antigen detection kits for the detection of both seasonal and AH1N1 pandemic 2009 influenza has been reported in laboratory or clinical studies [19, 20].

As of February 3, 2010, 225 oseltamivir-resistant cases were reported and confirmed worldwide [21]. All these oseltamivir-resistant isolates had the same mutation in the neuraminidase gene (H275Y). In Japan, the frequency of H275Y mutation was also very low for patients with 2009 pandemic influenza (1.10%: 76 of 6,916 analyzed viruses had the H275Y mutation) in a report on October 1, 2010 [22]. However, the situation could change in the future, because the rapid emergence of oseltamivir resistance was confirmed in a patient 4 days after early treatment with the standard dosage of oseltamivir for pandemic 2009 pneumonia [22, 23].

In conclusion, the reduced clinical effectiveness of oseltamivir against influenza in the 2008–2009 season, in which most isolated virus was seasonal H1N1 with the H275Y mutation, was not found in the 2009–2010 season, in which almost all isolated viruses were pandemic H1N1 2009. The effectiveness of zanamivir was unchanged over the three seasons. However, H275Y mutation related to oseltamivir resistance has occurred sporadically in the virus of patients with pandemic H1N1 virus infection in some parts of the world; thus, clinicians must bear in mind that oseltamivir resistance to this new influenza virus may

become more widespread, as has been seen with seasonal H1N1 virus, especially in immunocompromised or high-risk patients [25, 26]. In the 2009–2010 season, no seasonal influenza virus was detected, and oseltamivir and zanamivir were very effective in this study. However, we must continue careful surveillance because seasonal influenza H1N1 with the H275Y mutation may again become the most prevalent form in the near future, and 2009 pandemic H1N1 virus may acquire resistance to oseltamivir or zanamivir.

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References

1. 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;59:207–12.
2. 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;49:1825–35.
3. Tamura D, Mitamura K, Yamazaki M, Fujino M, Nirasawa M, Kimura K, et al. Oseltamivir-resistant influenza A viruses circulating in Japan. J Clin Microbiol. 2009;47:1424–7.
4. Infectious Disease Surveillance Center. Pandemic influenza A (H1N1) situation report of Japan, update 27, 25 December 2009. http://idsc.nih.go.jp/disease/swine_influenza_e/idsc_e2009/09idsc27e.html.
5. Dawood FS, Jain S, Finelli L, Shaw MW, Lindstrom S, Garten RJ, et al. Emergence of a novel swine-origin influenza A (H1N1) virus in humans. N Engl J Med. 2009;360:2605–15.
6. Cao B, Li XW, Mao Y, Wang J, Lu HZ, Chen YS, et al. Clinical features of the initial cases of 2009 pandemic influenza A (H1N1) virus infection in China. N Engl J Med. 2009;361:2507–17.
7. Lessler J, Reich NG, Cummings DA. Outbreak of 2009 pandemic influenza A (H1N1) at a New York City school. N Engl J Med. 2009;361:2628–36.
8. 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.
9. Kawai N, Ikematsu H, Iwaki N, Satoh I, Kawashima T, Tsuchimoto T, et al. A prospective, Internet-based study of the effectiveness and safety of influenza vaccination in the 2001–2002 influenza season. Vaccine. 2003;21:4507–13.
10. 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;43:439–44.
11. Kawai N, Ikematsu H, Iwaki N, Maeda T, Kanazawa H, Kawashima T, et al. A comparison of the effectiveness of zanamivir and oseltamivir for the treatment of influenza A and B. J Infect. 2008;56:51–7.
12. 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. Eurosurveillance. 2008;13:1–2.
13. Infectious Disease Surveillance Center. Weekly reports of influenza virus isolation/detection. 2010. https://hasseidoko.mhlw.go.jp/Byogentai/Pdf/data2e.pdf.
14. Libster R, Bugna J, Coviello S, Hijano DR, Dunaiewsky M, Reynoso N, et al. Pediatric hospitalizations associated with 2009 pandemic influenza A (H1N1) in Argentina. N Engl J Med. 2010;362:45–55.
15. Cauchemez S, Donnelly CA, Reed C, Ghani AC, Fraser C, Kent CK, et al. Household transmission of 2009 pandemic influenza A (H1N1) virus in the United States. N Engl J Med. 2009;361:2619–27.
16. Kawai N, Ikematsu H, Iwaki N, Maeda T, Kawashima T, Hirotsu N, et al. Comparison of the effectiveness of zanamivir and oseltamivir against influenza A/H1N1, A/H3N2, and B. Clin Infect Dis. 2009;48:996–7.
17. Cass LM, Brown J, Pickford M, Fayinka S, Newman SP, Johansson CJ, et al. Pharmacoscintigraphic evaluation of lung deposition of inhaled zanamivir in healthy volunteers. Clin Pharmacokinet. 1999;36(suppl 1):21–31.
18. WHO. Transmission dynamics and impact of pandemic influenza A(H1N1) 2009 virus. Weekly epidemiological record. 2009;46:481–84. (http://www.who.int/wer/2009/wer8446.pdf).
19. Sakai-Tagawa Y, Ozawa M, Tamura D, Le MQ, Nidom CA, Sugaya N, et al. Sensitivity of influenza rapid diagnostic tests to H5N1 and 2009 pandemic H1N1 viruses. J Clin Microbiol. 2010;48:2872–7.
20. Iwaki N, Ikematsu H, Kawai N, Hirotsu N, Tanaka O, Maeda T, et al. Usefulness of a self-blown nasal discharge specimen for use with immunochromatography based influenza antigen detection kits. In: Peiris M, editor. Options for the control of influenza VII. Atlanta: Integress; 2010. p. 185. (abstract book).
21. WHO. Update on oseltamivir-resistant pandemic A (H1N1) 2009 influenza virus. Weekly epidemiological record (WER). 2010;85:37–48. http://www.who.int/wer/2010/wer8506.pdf)
22. Infectious Disease Surveillance Center. Oseltamivir-resistant pandemic A (H1N1) 2009 influenza virus in Japan. http://idsc.nih.go.jp/iasr/graph/tamiful09-10.gif. Accessed 1 October 2010 (in Japanese).
23. Sy CL, Jung Lee SS, Liu MT, Tsai HC, Chen YS. Rapid emergence of oseltamivir resistance. Emerg Infect Dis. 2010;16:723–5.
24. 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;50:1252–5.
25. Gooskens J, Jonges M, Claas EC, Meijer A, van den Broek PJ, Kroes AM. Morbidity and mortality associated with nosocomial transmission of oseltamivir-resistant influenza A(H1N1) virus. JAMA. 2009;301:1042–6.
26. van der Vries E, van den Berg B, Schutten M. Fatal oseltamivir-resistant influenza virus infection. N Engl J Med. 2008;359:1074–6.

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Emergence of H274Y oseltamivir-resistant A(H1N1) influenza viruses in Japan during the 2008–2009 season

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ABSTRACT

Background: A substantial increase in oseltamivir-resistant A(H1N1) influenza viruses was reported in Europe in late 2007.
Objectives: To monitor the antiviral susceptibility profile of human A(H1N1) influenza viruses in Japan during the 2007–2008 and 2008–2009 seasons.
Study design: Viruses were obtained from respiratory samples of patients with influenza collected in Japan between December 2007 and April 2008 ($n = 1046$) and between December 2008 and April 2009 ($n = 1789$). Oseltamivir resistance was determined by an H274Y-specific real-time PCR cycling probe assay and a neuraminidase inhibition assay. Amantadine resistance was assessed by sequencing the M2 gene. Sequencing of the hemagglutinin and NA genes was performed to infer phylogenetic relationships between different strains.
Results: Three of 687 (0.4%) A(H1N1) viruses from the 2007–2008 season and 745 of 745 (100%) viruses from the 2008–2009 season carried the NA–H274Y substitution and demonstrated a >300-fold reduction in oseltamivir susceptibility. All oseltamivir-resistant viruses from the 2008–2009 season possessed an A193T substitution in the receptor-binding domain of the hemagglutinin. Amantadine resistance was detected in 431 of 687 (62.7%) and 0 of 745 (0.0%) of the A(H1N1) viruses from the 2007–2008 and 2008–2009 seasons, respectively.
Conclusions: A dramatic surge in oseltamivir-resistant A(H1N1) viruses possessing the NA–H274Y substitution was detected in Japan during the 2008–2009 season. The emergence of oseltamivir-resistant viruses was facilitated by mutations in the viral genome. Intensified surveillance, including phenotypic assays and sequencing of the hemagglutinin, neuraminidase, and M2 gene would allow monitoring of the spread and evolution of drug-resistant influenza virus variants.

1. Background

The neuraminidase inhibitors (NAI) oseltamivir and zanamivir are currently the drugs of choice against influenza.[1] Until recently, surveillance studies have revealed a low prevalence (approximately 1%) of NAI resistance among influenza A and B viruses circulating worldwide.[2] However, a substantial increase in oseltamivir-resistant A(H1N1) viruses was reported during the 2007–2008 season, with the highest resistance rate (66%) detected in Europe.[3] And oseltamivir-resistant A(H1N1) viruses were the dominant circulating strain during the 2008 season in the Southern Hemisphere.[4,5] All reported cases of recent oseltamivir-resistant viruses have a histidine (H) to tyrosine(Y) substitution at position 274 (H274Y) of the neuraminidase (NA), a substitution that is known to confer a high level of resistance to oseltamivir but not to zanamivir.[6–9] In contrast, in Japan, in the country with the world's highest per capita use of oseltamivir, the prevalence of oseltamivir-resistant A(H1N1) viruses with the H274Y substitution remained low (1.5–2.6%) during the 2007–2008 season.[10,11]

Abbreviations: NAI, neuraminidase inhibitors; H, histidine; Y, tyrosine; NA, neuraminidase; MDCK, Madin Darby canine kidney; MUNANA, methyl umbelliferone *N*-acetyl neuraminic acid; HA, hemagglutinin.

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2. Objectives

In the present study, we monitored the emergence and spread of oseltamivir-resistant H274Y A(H1N1) influenza viruses in multiple areas in Japan during the 2007–2008 and 2008–2009 seasons, and detected a dramatic surge in oseltamivir resistance during the 2008–2009 season.

3. Study design

Physician-based sentinel surveillance was conducted among 2835 pediatric and adult patients who tested positive by a rapid influenza diagnostic kit such as the Quick-Ex Flu kit (Denka Seiken, Japan) in seven prefectures (Hokkaido, Niigata, Gunma, Kyoto, Hyogo, Tottori, and Nagasaki) in Japan between December 2007 and April 2008 ($n = 1046$) and between December 2008 and April 2009 ($n = 1789$).

3.1. Sample collection

An informed consent was obtained from patients or patient's guardians. Basic demographic information (sex and age) and a recent history of anti-influenza treatment were recorded by clinicians. Sampling was done prior to initiating any antiviral drug treatment. Nasopharyngeal swabs were obtained, placed in viral transport media, and transported to the Division of Public Health at the Niigata University for virus isolation. This study was approved by the medical faculty ethics committee of the Niigata University Graduate School of Medical and Dental Sciences.

3.2. Virus isolation

Initial isolation of influenza viruses was performed using Madin Darby canine kidney (MDCK) cells, as previously described.[12] Influenza isolates were typed and subtyped by a hemagglutination inhibition assay using guinea pig red blood cells and commercially available influenza vaccine strain antisera (Denka Seiken Co., Ltd., Tokyo, Japan) for the 2007–2008 season in Japan [A/Solomon Islands/3/2006 (H1N1), A/Hiroshima/52/2008 (H3N2), B/Malaysia/2506/2004], and for the 2008–2009 season [(A/Brisbane/59/2007 (H1N1), A/Uruguay/716/2007 (H3N2), B/Florida/4/2006].

3.3. RNA extraction

RNA was extracted from 100 μl of each influenza isolate using the Extragen II kit (Kainos, Tokyo, Japan). Complementary DNA synthesis was performed using the generic influenza A primer, uni12, as described elsewhere.[13]

3.4. Screening for the H274Y substitution in the NA gene conferring oseltamivir-resistance

The detection of single-nucleotide polymorphisms in the NA gene was performed using a real-time PCR cycling probe assay.[14] A(H1N1)-specific primers and fluorescent dye-labeled DNA–RNA chimeric probes were designed to detect a C→T mutation at nucleotide position 763, a mutation that results in the substitution of histidine (encoded by CAC) for tyrosine (encoded by TAC) at amino acid position 274 in the NA protein (protocol available on request).

3.5. Screening for substitutions in the M2 gene conferring amantadine-resistance

Direct sequencing was carried out using M2 gene-specific primers, as described previously.[15] PCR products were sequenced

Table 1
Number of influenza A(H1N1) viruses isolated in various areas in Japan during the 2007–2008 and 2008–2009 influenza seasons and their antiviral susceptibilities.

Prefecture	2007–2008 influenza season				2008–2009 influenza season			
	No. of A(H1N1) isolates/total no. of isolates per season	Proportion of A(H1N1) viruses circulating (%)	No. of H274Y mutants (%)[a]	No. of S31N mutants (%)[b]	No. of A(H1N1) isolates/total no. of isolates per season	Proportion of A(H1N1) viruses circulating (%)	No. of H274Y mutants (%)[a]	No of S31N mutants (%)[b]
Hokkaido	11/11	100.0	0/11 (0.0)	0/11 (0.0)	22/24	95.6	22/22 (100.0)	0/22 (0.0)
Niigata	74/87	85.1	0/74 (0.0)	54/74 (73.0)	283/510	58.0	283/283 (100.0)	0/283 (0.0)
Gunma	11/13	84.6	0/11 (0.0)	8/11 (72.7)	7/19	36.8	7/7 (100.0)	0/7 (0.0)
Kyoto	385/420	91.7	0/385 (0.0)	239/385 (62.1)	243/480	54.6	243/243 (100.0)	0/243 (0.0)
Hyogo	95/96	99.0	1/95 (1.1)	74/95 (77.9)	58/83	69.9	58/58 (100.0)	0/58 (0.0)
Tottori	NA	NA	NA	NA	43/117	36.8	3/43 (100.0)	0/43 (0.0)
Nagasaki	111/145	76.6	2/111 (1.8)	56/111 (50.5)	89/131	67.9	89/89 (100.0)	0/89 (0.0)
Total	687/773	88.9	3/687 (0.4)	431/687 (62.7)	745/1364	54.6	745/745 (100.0)	0/745 (0.0)

NA, not available. Clinics from Tottori Prefecture jointed the study as of December 2008.
[a] Genotyped by H274Y-specific real-time PCR assay.
[b] Genotyped by the direct sequencing of a short region (from 680 to 910 bp) of the M2 gene associated with amantadine resistance.

using BigDye Terminator v 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and an ABI 3100 automated sequencer (Applied Biosystems) according to the manufacturer's protocols (ABI, Boston).

3.6. NA inhibition assay

The susceptibility to oseltamivir carboxylate and zanamivir was examined by a previously described fluorescence-based NA inhibition assay using methyl umbelliferone *N*-acetyl neuraminic acid (MUNANA) as the substrate.[16] Twenty-three and 94 A(H1N1) viruses isolated during the 2007–2008 and 2008–2009 seasons, respectively, were tested. The reference viruses A/Texas/36/91 (H1N1) with the H274Y substitution in the NA gene and wild-type oseltamivir-sensitive virus A/Texas/36/91V40 (H1N1) (Y274), which were kindly provided by Gubareva L.V., were included in each independent assay. Zanamivir and oseltamivir carboxylate, were provided by GlaxoSmithKline (Brentford, United Kingdom), and Roche Products, Ltd. (Basel, Switzerland), respectively.

3.7. Sequence analysis of the HA and NA genes

Sequencing was performed using previously reported primers for the 47 and 53 randomly selected influenza A(H1N1) isolates from the 2007–2008 and 2008–2009 seasons, respectively.[17] The NA and HA sequences were edited and assembled using DNASTAR Lasergene 7 (Bioinformatics Pioneer DNASTAR, Inc., Wisconsin, USA), and phylogenetic trees were constructed by the neighbor-joining method[18] and bootstrap analysis ($n = 1000$) using MEGA 4.0 software.[19] Corresponding amino acid mutations in the HA and NA are specified according to H3 and N2 numbering, respectively.[20] The WHO recommended vaccine strains and other reference strains included in the phylogenetic trees were downloaded from the Influenza Virus Resource database.[21]

3.8. Nucleotide sequence accession numbers

The GenBank Database accession numbers of the HA and NA nucleotide sequences obtained in this study are listed in Appendix Table A1.

4. Results

4.1. Virus isolation and patient demographics

A total of 773 and 1364 influenza viruses were isolated during the 2007–2008 and 2008–2009 seasons, respectively (Table 1). Overall, influenza A(H1N1) viruses accounted for 687 of 773 (89%) viruses collected during the 2007–2008 season and for 745 of 1364 (55%) viruses collected during the 2008–2009 season. The remaining viruses were influenza A(H3N2) viruses, accounting for 61 of 773 (8%) and 334 of 1364 (24%) viruses in the 2007–2008 and 2008–2009 seasons, respectively, and influenza B viruses, accounting for 25 of 773 (3%) and 285 of 1364 (21%) viruses in the 2007–2008 and 2008–2009 seasons, respectively.

Demographic data were available for 671 of 687 (98%) patients with A(H1N1) infection in the 2007–2008 season and for 444 of 745 (60%) patients with A(H1N1) infection in the 2008–2009 season. The median age of patients infected with oseltamivir-resistant A(H1N1) viruses was 27.83 years (range 6.42–36.75, $n = 3$) and 8.46 years (range 0.5–87.25, $n = 444$) for the 2007–2008 and 2008–2009 seasons, respectively. Male patients were the source of three (100%) and 235 (53%) oseltamivir-resistant isolates during the 2007–2008 and 2008–2009 seasons, respectively. None of patients infected with oseltamivir-resistant A(H1N1) viruses reported a history of

Table 2
IC_{50} values in the neuraminidase inhibition assay for A(H1N1) isolates collected in Japan during the 2007–2008 and 2008–2009 seasons.

	Control viruses		2007–2008 influenza season		2008–2009 influenza season	
	H274 virus[a] (A/Texas/36/91V40)	H274Y virus[b] (A/Texas/36/91P)	H 274 viruses, $n = 20$ (range)	H274Y viruses, $n = 3$ (range)	H 274 viruses, $n = 0$ (range)	H 274 viruses, H274Y viruses, $n = 94$ (range)
Oseltamivir carboxylate IC_{50} [nM]	2.17	848.97	2.34 (1.26–5.01)	937.84 (901.22–1253.90)	–[c]	953.39 (610.76–2712.50)
Zanamivir IC_{50} [nM]	0.96	1.03	1.79 (0.66–3.42)	1.35 (1.06–2.44)	–[c]	1.58 (0.63–5.95)

[a] Viruses without mutation at position 274 in the neuraminidase protein.
[b] Viruses that have a histidine (H) to tyrosine (Y) mutation at position 274 of the neuraminidase protein.
[c] No isolates with H274 were detected during the 2008–2009 season.

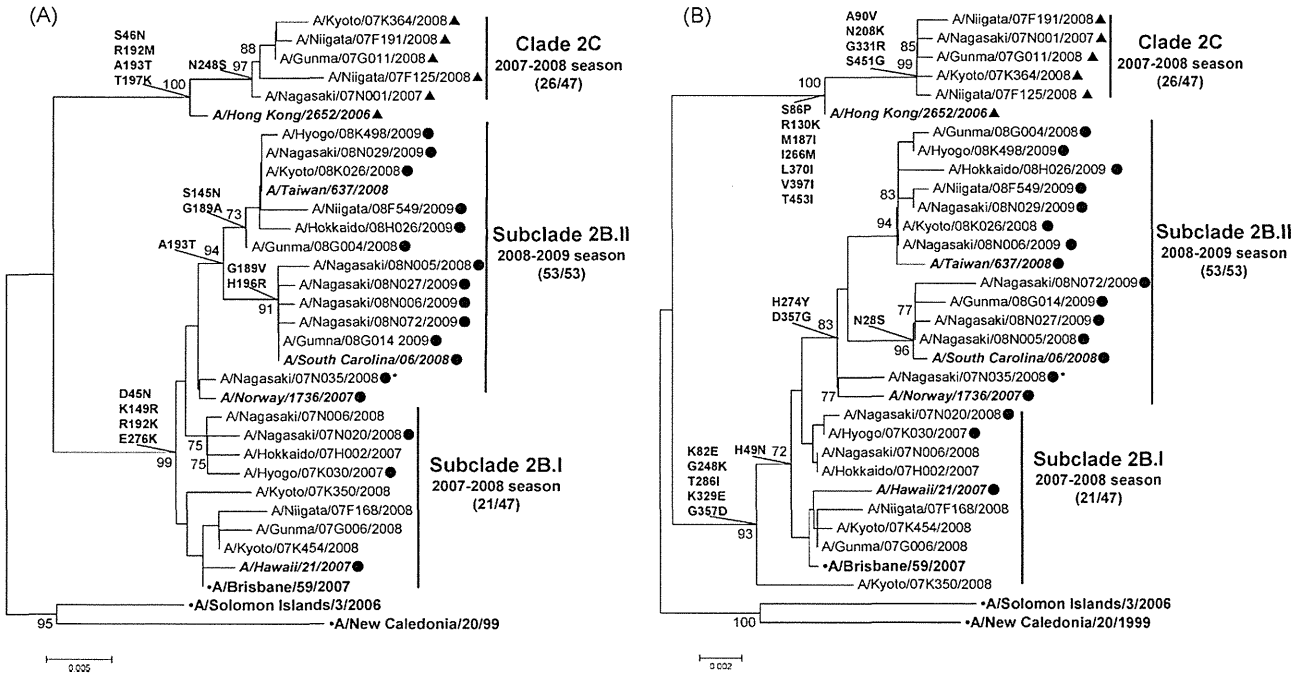

Fig. 1. Phylogenetic comparison of the hemagglutinin (A) and neuraminidase (B) genes of human influenza A(H1N1) during 2007–2008 and 2008–2009 seasons in Japan. Only selected sequences are shown in the figure. Closed triangle (▲) represents amantadine-resistant strains exhibiting the M2 S31N substitution. Oseltamivir-resistant strains exhibiting the NA H247Y substitution are denoted with (●). Vaccine strains are in bold; reference strains are in *bold italic*. The proportion in parenthesis represents the number of sequences that belonged to the corresponding clade out of total sequenced strains at that season. Phylogenetic trees were inferred from 830 nucleotide sequences for the hemagglutinin, HA (HA1 subunit) and 1372 nucleotide for the neuraminidase, NA by the neighbor-joining method. Bootstrap values of more than 70% are shown. Common amino acid changes that distinguish Clade 2 from subgroups of Clade 2 are indicated. *Except for the A/Nagasaki/0735/2008 strain that was isolated in the 2007–2008 season.

anti-influenza treatment (including amantadine, oseltamivir, and zanamivir) before testing for influenza.

4.2. Genotypic antiviral resistance

Three of the 687 (0.4%) influenza A(H1N1) isolates from the 2007–2008 season and all the 745 (100%) influenza A(H1N1) isolates from the 2008–2009 season possessed the H274Y substitution in the NA, which confers oseltamivir-resistance. Amantadine-resistance S31N substitution in the M2 gene was detected in 431 of the 687 (62.7%) influenza A(H1N1) isolates from the 2007–2008 season but in none of the 745 (0.0%) influenza A(H1N1) isolates from the 2008–2009 season (Table 1).

4.3. NAI susceptibility testing

The median oseltamivir carboxylate IC_{50} values for A(H1N1) viruses carrying the H274Y substitution were 937.84 and 953.39 nM for the 2007–2008 and 2008–2009 viruses, respectively (Table 2). The H274Y viruses demonstrated a roughly 300–400-fold reduction in oseltamivir susceptibility compared to the wild-type reference strain A/Texas/36/91P. None of the isolates demonstrated reduced susceptibility to zanamivir.

4.4. Phylogenetic analysis of the HA and NA genes

In the HA and NA phylogenies, viruses from the 2007–2008 and 2008–2009 seasons belonged to two clades: 2C and 2B (Fig. 1). Clade 2C included 26 of 47 (55%) isolates from the 2007–2008 season; all of these viruses were amantadine-resistant and oseltamivir-sensitive. Clade 2B was further divided into two subclades, depending on the NA phylogeny. Subclade 2B.I, referred to as the "Hawaiian" lineage,[22] included 21 of 47 (45%) isolates from the 2007–2008 season, all of which were amantadine- and oseltamivir-sensitive, except for two amantadine-sensitive but oseltamivir-resistant viruses (A/Nagasaki/07N020/2008 and Hyogo/07K30/2007).

Subclade 2B.II, referred to as the "Northern European" lineage,[22] included all (53/53) isolates from the 2008–2009 season and one (A/Nagasaki/07N035/2008) from the 2007–2008 season. All of these viruses were amantadine-sensitive but oseltamivir-resistant and possessed the H274Y and D357G substitutions in the NA. Compared to viruses of "Northern European" lineage that circulated during the 2007–2008 season, including our A/Nagasaki/07N006/2008 virus, subclade 2B.II viruses from the 2008–2009 season possessed an additional A193T substitution in the receptor-binding domain of HA. In addition, isolates of subclade 2B.II had polymorphisms at positions 189 (G189A and G189V), 145 (S145N), and 196 (H196R) in the HA (Fig. 1).

5. Discussion

In the present study we demonstrated the emergence and extensive spread of oseltamivir-resistant influenza A(H1N1) viruses carrying the H274Y substitution in the NA in Japan during the 2008–2009 season. The prevalence of oseltamivir-resistant A(H1N1) isolates in Japan increased from 0.4% in the 2007–2008 season to 100% in the 2008–2009 season and was universal in all seven prefectures studied. Importantly, all oseltamivir-resistant viruses remain susceptible to zanamivir and amantadine.

In Japan, the number of per capita oseltamivir prescriptions is 70.9/1000 inhabitants/year, which accounts for 75% of all prescriptions in the world.[23,24] Nevertheless, our results, in agreement with previous reports, indicate a low frequency (0.4–2.6%) of oseltamivir resistance in Japan for patients not treated with NAIs in the 2007–2008 season.[10,11] In contrast, the 2007–2008 season was marked by a striking increase in the prevalence of oseltamivir-resistant A(H1N1) viruses in Northern Europe, the US, and South

Africa.[3–5,25,26] As oseltamivir is rarely used in South Africa and the number of oseltamivir courses sold in the Northern Europe is 400 times lower (0.17–1.64/1000 inhabitants/year)[27] than in Japan, it is unlikely that drug selection pressure is the main factor contributing to the rapid rise of oseltamivir-resistant influenza viruses. Instead, the A193T substitution in the HA1, belonging to receptor-binding domain and to antigenic site Sb,[28] might play a role in fixation of oseltamivir-resistant lineage in the population by a similar non-drug induced mechanism as for emergence of amantadine-resistance viruses.[29] The A193T substitution could also help oseltamivir-resistant viruses to evade previously acquired host immunity against A/Brisbane/59/2007-like viruses, such as Clade 2B.I viruses and Clade 2B.II viruses of the "Northern European" prototype, which circulated in the previous season. It is also likely that other mutations located elsewhere in the viral genome contributed to the enhanced fitness of the H274Y isolates from the 2008–2009 season. Therefore, whole-genome sequencing studies would be desirable to identify other advantageous mutations.

Amantadine-resistant influenza A(H1N1) viruses first emerged in Japan during the 2006–2007 season[29] and continued circulating during the following 2007–2008 season in which amantadine-resistant viruses predominated over amantadine-sensitive viruses (this study). In the present study, we also monitored a remarkable drop in amantadine resistance from 66% in the 2007–2008 season to 0% in the 2008–2009 season. This result implies that H274Y viruses might have a superior fitness over amantadine-resistant influenza A(H1N1) viruses, which have been circulating in humans for two successive seasons.

It was suggested that influenza A(H3N2) viruses usually originate in South Asia and towards Europe, the North America and South America.[30] In contrast, the newly emerged oseltamivir-resistant A(H1N1) lineage was first detected in Europe. The surveillance data on A(H1N1) virus circulation in Vietnam and Southeastern China shows that only a small number of H274Y influenza A(H1N1) circulated during the 2007–2008 season.[31] This suggests that a new influenza lineage could emerge anywhere in the world and might provide an evidence for a difference in the circulation patterns of influenza A(H1N1) and A(H3N2) viruses.

The emergence of oseltamivir resistance and the reversion to amantadine sensitivity in A(H1N1) viruses emphasize the importance of monitoring the antiviral susceptibility profile of influenza viruses at a local level to determine the appropriate antiviral treatment. Surveillance studies that combine rapid detection technology such as the real-time PCR cycling probe method, phenotypic assays, and sequence data for HA, NA, and M2 genes would permit monitoring of the spread and evolution of antiviral-resistant influenza virus variants, allowing for a prompt response.

Conflicts of interest

The authors have no conflicts of interest to disclose.

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Appendix A.

Table A1
GenBank accession numbers of hemagglutinin and neuraminidase sequences.

Strain	Hemagglutinin	Neuraminidase
A/Gunma/07G001/2007	CY043580	CY043581
A/Gunma/07G011/2008	CY043582	CY043583
A/Gunma/07G019/2008	CY043584	CY043585
A/Hokkaido/07H002/2007	CY043586	CY043587
A/Hokkaido/07H012/2007	CY043588	CY043589
A/Kyoto/07K001/2007	CY043590	CY043591
A/Kyoto/07K004/2007	CY043592	CY043593
A/Kyoto/07K008/2008	CY043594	CY043595
A/Kyoto/07K018/2008	CY043596	CY043597
A/Kyoto/07K088/2008	CY043602	CY043603
A/Kyoto/07K112/2008	CY043604	CY043605
A/Kyoto/07K145/2008	CY043606	CY043607
A/Kyoto/07K225/2008	CY043608	CY043609
A/Kyoto/07K232/2008	CY043610	CY043611
A/Kyoto/07K350/2008	CY043612	CY043613
A/Kyoto/07K364/2008	CY043614	CY043615
A/Kyoto/07K415/2008	CY043616	CY043617
A/Kyoto/07K431/2008	CY043618	CY043619
A/Kyoto/07K466/2008	CY043620	CY043621
A/Kyoto/07K520/2008	CY043622	CY043623
A/Hyogo/07K036/2008	CY043598	CY043599
A/Hyogo/07K084/2008	CY043600	CY043601
A/Nagasaki/07N001/2007	CY043624	CY043625
A/Nagasaki/07N006/2008	CY043626	CY043627
A/Nagasaki/07N010/2008	CY043628	CY043629
A/Nagasaki/07N014/2008	CY043630	CY043631
A/Nagasaki/07N016/2008	CY043632	CY043633
A/Niigata/07F006/2008	CY043566	CY043567
A/Niigata/07F042/2008	CY043568	CY043569
A/Niigata/07F071/2008	CY043570	CY043571
A/Niigata/07F081/2008	CY043572	CY043573
A/Niigata/07F144/2008	CY043574	CY043575
A/Niigata/07F168/2008	CY043576	CY043577
A/Niigata/07F203/2008	CY043578	CY043579

Table A1 (*Continued*)

Strain	Hemagglutinin	Neuraminidase
A/Gunma/08G004/2008	CY043654	CY043655
A/Gunma/08G005/2008	CY043656	CY043657
A/Gunma/08G011/2009	CY043658	CY043659
A/Gumna/08G014/2009	CY043660	CY043661
A/Hokkaido/08H003/2008	CY043662	CY043663
A/Hokkaido/08H006/2008	CY043664	CY043665
A/Hokkaido/08H009/2008	CY043666	CY043667
A/Hokkaido/08H015/2009	CY043668	CY043669
A/Hokkaido/08H017/2009	CY043670	CY043671
A/Hokkaido/08H026/2009	CY043672	CY043673
A/Hokkaido/08H033/2009	CY043674	CY043675
A/Hyogo/08K416/2009	CY043692	CY043693
A/Hyogo/08K498/2009	CY043694	CY043695
A/Hyogo/08K500/2009	CY043696	CY043697
A/Kyoto/08K008/2008	CY043676	CY043677
A/Kyoto/08K026/2008	CY043678	CY043679
A/Kyoto/08K072/2008	CY043680	CY043681
A/Kyoto/08K098/2009	CY043682	CY043683
A/Kyoto/08K250/2009	CY043684	CY043685
A/Kyoto/08K273/2009	CY043686	CY043687
A/Kyoto/08K281/2009	CY043688	CY043689
A/Kyoto/08K410/2009	CY043690	CY043691
A/Nagasaki/08N001/2008	CY043698	CY043699
A/Nagasaki/08N004/2008	CY043700	CY043701
A/Nagasaki/08N005/2008	CY043702	CY043703
A/Nagasaki/08N011/2009	CY043704	CY043705
A/Nagasaki/08N024/2009	CY043706	CY043707
A/Nagasaki/08N026/2009	CY043708	CY043709
A/Nagasaki/08N027/2009	CY043710	CY043711
A/Nagasaki/08N029/2009	CY043712	CY043713
A/Nagasaki/08N072/2009	CY043714	CY043715
A/Niigata/08F002/2009	CY043634	CY043635
A/Niigata/08F009/2009	CY043636	CY043637
A/Niigata/08F036/2009	CY043638	CY043639
A/Niigata/08F067/2009	CY043640	CY043641
A/Niigata/08F306/2009	CY043642	CY043643
A/Niigata/08F483/2009	CY043644	CY043645
A/Niigata/08F491/2009	CY043646	CY043647
A/Niigata/08F549/2009	CY043648	CY043649
A/Niigata/08F631/2009	CY043650	CY043651
A/Niigata/08F717/2009	CY043652	CY043653
A/Tottori/08T001/2008	CY043716	CY043717
A/Tottori/08T027/2009	CY043718	CY043719
A/Tottori/08T047/2009	CY043720	CY043721
A/Tottori/08T099/2009	CY043722	CY043723
A/Tottori/08T121/2009	CY043724	CY043725
A/Tottori/08T125/2009	CY043726	CY043727

References

1. Moscona A. Neuraminidase inhibitors for influenza. *N Engl J Med* 2005;**353**:1363–73.
2. Sheu T, Deyde V, Okomo-Adhiambo M, et al. Surveillance for neuraminidase inhibitor resistance among human influenza A and B viruses circulating worldwide from 2004 to 2008. *Antimicrob Agents Chemother* 2008;**52**:3284–92.
3. Meijer A, Lackenby A, Hungnes O, et al. Oseltamivir-resistant influenza virus A (H1N1) Europe, 2007–2008 season. *Emerg Infect Dis* 2009;**15**:552–60.
4. Besselaar T, Naidoo D, Buys A, et al. Widespread oseltamivir resistance in influenza A viruses (H1N1) in South Africa. *Emerg Infect Dis* 2008;**14**:1809–10.
5. Hurt A, Ernest J, Deng Y, et al. Emergence and spread of oseltamivir-resistant A(H1N1) influenza viruses in Oceania South East Asia and South Africa. *Antiviral Res* 2009;**83**:90–3.
6. Gubareva L, Kaiser L, Matrosovich M, et al. Selection of influenza virus mutants in experimentally infected volunteers treated with oseltamivir. *J Infect Dis* 2001;**183**:523–31.
7. Ives J, Carr J, Mendel D, et al. The H274Y mutation in the influenza A/H1N1 neuraminidase active site following oseltamivir phosphate treatment leave virus severely compromised both in vitro and in vivo. *Antiviral Res* 2002;**55**:307–17.
8. Wang M, Tai C, Mendel D. Mechanism by which mutations at his274 alter sensitivity of influenza a virus n1 neuraminidase to oseltamivir carboxylate and zanamivir. *Antimicrob Agents Chemother* 2002;**46**:3809–16.
9. Abed Y, Goyette N, Boivin G. A reverse genetics study of resistance to neuraminidase inhibitors in an influenza A/H1N1 virus. *Antivir Ther* 2004;**9**:577–81.
10. Tamura D, Mitamura K, Yamazaki M, et al. Oseltamivir-resistant influenza a viruses circulating in Japan. *J Clin Microbiol* 2009;**47**:1424–7.
11. Pediatric advisory committee briefing document for tamiflu. Tamiflu (RO 64-0796) PAC briefing document; 2008. Available at: http://www.fda.gov/ohrms/dockets/AC/07/briefing/2007-4325b_02_15_Sponsor%20Background%20Package%20Roche.pdf. Accessed 3 August 2009.
12. Saito R, Li D, Suzuki Y, et al. High prevalence of amantadine-resistance influenza a (H3N2) in six prefectures Japan, in the 2005–2006 season. *J Med Virol* 2007;**79**:1569–76.
13. Hoffmann E, Stech J, Guan Y, et al. Universal primer set for the full-length amplification of all influenza A viruses. *Arch Virol* 2001;**146**:2275–89.
14. Esaki H, Noda K, Otsuki N, et al. Rapid detection of quinolone-resistant Salmonella by real time SNP genotyping. *J Microbiol Methods* 2004;**58**:131–4.
15. Masuda H, Suzuki H, Oshitani H, et al. Incidence of amantadine-resistant influenza A viruses in sentinel surveillance sites and nursing homes in Niigata, Japan. *Microbiol Immunol* 2000;**44**:833–9.
16. Hurt A, Barr I, Hartel G, et al. Susceptibility of human influenza viruses from Australasia and South East Asia to the neuraminidase inhibitors zanamivir and oseltamivir. *Antiviral Res* 2004;**62**:37–45.
17. Dapat C, Saito R, Kyaw Y, Naito M, Hasegawa G, Suzuki Y, et al. Epidemiology of human influenza A and B viruses in Myanmar from 2005 to 2007. *Intervirology* 2009;**52**:310–20.
18. Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* 1987;**4**:406–25.
19. Kumar S, Tamura K, Nei M. MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. *Brief Bioinform* 2004;**5**:150–63.
20. Varghese J, Colman P. Three-dimensional structure of the neuraminidase of influenza virus A/Tokyo/3/67 at 2.2 A resolution. *J Mol Biol* 1991;**221**:473–86.
21. Bao Y, Bolotov P, Dernovoy D, et al. The influenza virus resource at the National Center for Biotechnology Information. *J Virol* 2008;**82**:596–601.
22. Kawakami C, Obuchi M, Saikusa M, et al. Isolation of oseltamivir-resistant influenza A/H1N1 virus of different origins in Yokohama City Japan, during the 2007–2008 influenza season. *Jpn J Infect Dis* 2009;**62**:83–6.
23. Yasui K, Amano Y, Minami I, et al. Recent changes in the trends of seasonal influenza outbreaks in the Nagano Prefectural area of Japan: an oseltamivir effect? *J Infect Chemother* 2007;**13**:429–31.
24. The Infectious Diseases Surveillance Center. 2007/2008 influenza season, Japan. Infectious agents surveillance report 2008;29:297–9. Available at: http://idsc.nih.go.jp/iasr/29/345/tpc345.html. Accessed 3 August 2009.
25. Jonges M, van der Lubben I, Dijkstra F, et al. Dynamics of antiviral-resistant influenza viruses in the Netherlands, 2005–2008. *Antiviral Res* 2009;**83**:290–7.
26. Dharan N, Gubareva L, Meyer J, et al. Infections with oseltamivir-resistant influenza A(H1N1) virus in the United States. *JAMA* 2009;**301**:1034–41.
27. Kramarz P, Monnet D, Nicoll A, et al. Use of oseltamivir in 12 European countries between 2002 and 2007—lack of association with the appearance of oseltamivir-resistant influenza A(H1N1) viruses. *Eur Surveill* 2009:14.
28. Gamblin S, Haire L, Russell R, et al. The structure and receptor binding properties of the 1918 influenza hemagglutinin. *Science* 2004;**303**:1838–42.
29. Saito R, Suzuki Y, Li D, et al. Increased incidence of adamantane-resistant influenza A(H1N1) and A(H3N2) viruses during the 2006–2007 influenza season in Japan. *J Infect Dis* 2008;**197**:630–2; [author reply 2–3].
30. Russell C, Jones T, Barr I, et al. The global circulation of seasonal influenza A (H3N2) viruses. *Science* 2008;**320**:340–6.
31. WHO. Influenza A(H1N1) virus resistance to oseltamivir, 2008. Available at: http://www.who.int/csr/disease/influenza/ResistanceTable200806013.pdf. Accessed 27 July 2009.

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Genetic Makeup of Amantadine-Resistant and Oseltamivir-Resistant Human Influenza A/H1N1 Viruses

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Genetic Makeup of Amantadine-Resistant and Oseltamivir-Resistant Human Influenza A/H1N1 Viruses[▽]

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The emergence and widespread occurrence of antiviral drug-resistant seasonal human influenza A viruses, especially oseltamivir-resistant A/H1N1 virus, are major concerns. To understand the genetic background of antiviral drug-resistant A/H1N1 viruses, we performed full genome sequencing of prepandemic A/H1N1 strains. Seasonal influenza A/H1N1 viruses, including antiviral-susceptible viruses, amantadine-resistant viruses, and oseltamivir-resistant viruses, obtained from several areas in Japan during the 2007–2008 and 2008–2009 influenza seasons were analyzed. Sequencing of the full genomes of these viruses was performed, and the phylogenetic relationships among the sequences of each individual genome segment were inferred. Reference genome sequences from the Influenza Virus Resource database were included to determine the closest ancestor for each segment. Phylogenetic analysis revealed that the oseltamivir-resistant strain evolved from a reassortant oseltamivir-susceptible strain (clade 2B) which circulated in the 2007–2008 season by acquiring the H275Y resistance-conferring mutation in the NA gene. The oseltamivir-resistant lineage (corresponding to the Northern European resistant lineage) represented 100% of the H1N1 isolates from the 2008–2009 season and further acquired at least one mutation in each of the polymerase basic protein 2 (PB2), polymerase basic protein 1 (PB1), hemagglutinin (HA), and neuraminidase (NA) genes. Therefore, a reassortment event involving two distinct oseltamivir-susceptible lineages, followed by the H275Y substitution in the NA gene and other mutations elsewhere in the genome, contributed to the emergence of the oseltamivir-resistant lineage. In contrast, amantadine-resistant viruses from the 2007–2008 season distinctly clustered in clade 2C and were characterized by extensive amino acid substitutions across their genomes, suggesting that a fitness gap among its genetic components might have driven these mutations to maintain it in the population.

Seasonal outbreaks of influenza cause substantial morbidity and mortality and significant economic losses each year (33). Periodically, new strains emerge in humans and cause pandemics that pose a great threat to human health (31). Vaccines are very important for the prevention of infection with influenza virus, but antiviral drugs remain essential for treatment as well as prophylaxis. Two classes of antiviral drugs with activity against the influenza virus are available: the M2 ion channel blockers, or adamantanes (rimantadine and amantadine), and the neuraminidase inhibitors (NAIs; oseltamivir and zanamivir) (22, 39).

The rapid surge in amantadine-resistant influenza A/H3N2 viruses since the 2003–2004 season and among H1N1 viruses in the 2005–2006 season is a great concern to the medical and public health communities (3, 8, 29, 38). Remarkably, while amantadine-resistant A/H3N2 viruses swiftly replaced susceptible viruses and have become dominant since then, amantadine-resistant A/H1N1 viruses could outcompete susceptible viruses during only two successive seasons (2006–2007 and 2007–2008) and retreated during the 2008–2009 season (2, 5, 29, 34). Nonetheless, an oseltamivir-resistant A/H1N1 strain, referred to as the Northern Europe lineage, emerged in the 2007–2008 season and eventually prevailed in Europe (comprising 68% of A/H1N1 viruses collected) and the southern hemisphere and later became predominant in many countries, including Japan, during the 2008–2009 season (9, 13, 21, 41).

Antiviral resistance is conferred by a single amino acid substitution in the target protein. Almost all amantadine-resistant viruses of both the A/H1N1 and the A/H3N2 subtypes have a serine-to-asparagine mutation at position 31 (S31N) of the M2 ion channel protein (14, 29, 30, 32), and the oseltamivir-resistant A/H1N1 strain has a histidine-to-tyrosine mutation at position 275 (H275Y, N1 numbering) of the neuraminidase (NA) protein (9, 13, 21). While mutations in target proteins are usually selected by drug pressure, drug selection alone does not seem to be the sole driving force for the establishment of an efficiently replicating and transmissible strain (9, 19, 32). This notion is supported by the fact that a high proportion of oseltamivir-resistant strains, namely, strains of the Northern Europe lineage, was first observed in Europe, where the level of NAI consumption is generally low (19, 21, 26), while Japan, which has been using more oseltamivir than the rest of the world, detected high proportions of resistant viruses 1 year later (35, 40). It is important to note that oseltamivir-resistant viruses were detected earlier but remained sporadic and could not prevail like the Northern Europe resistant lineage (18). Thus, compensating mutations occurring elsewhere in the genome were suggested to improve the fitness and transmissibility of resistant viruses (9, 21).

To address this point, in the study described here, we per-

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TABLE 1. Influenza A/H1N1 viruses sequenced in this study

Virus	Season	Isolation date (day/mo/yr)	Antiviral resistance
A/Gunma/07G002/2008	2007–2008	05/01/2008	Amantadine
A/Gunma/07G006/2008	2007–2008	28/01/2008	NA[a]
A/Hokkaido/07H007/2007	2007–2008	10/12/2007	NA
A/Hyogo/07K030/2007	2007–2008	18/12/2007	Oseltamivir
A/Kyoto/07K303/2008	2007–2008	21/01/2008	Amantadine
A/Kyoto/07K316/2008	2007–2008	28/01/2008	NA
A/Kyoto/07K454/2008	2007–2008	26/02/2008	NA
A/Nagasaki/07N005/2008	2007–2008	07/01/2008	Amantadine
A/Nagasaki/07N011/2008	2007–2008	20/02/2008	NA
A/Nagasaki/07N020/2008	2007–2008	14/01/2008	Oseltamivir
A/Nagasaki/07N035/2008	2007–2008	20/02/2008	Oseltamivir
A/Niigata/07F102/2008	2007–2008	18/02/2008	NA
A/Niigata/07F125/2008	2007–2008	21/02/2008	Amantadine
A/Niigata/07F191/2008	2007–2008	08/03/2008	Amantadine
A/Yokohama/78/2008	2007–2008	28/02/2008	Oseltamivir
A/Gunma/08G006/2009	2008–2009	05/01/2009	Oseltamivir
A/Hokkaido/08H024/2009	2008–2009	13/01/2009	Oseltamivir
A/Kyoto/08K056/2009	2008–2009	06/01/2009	Oseltamivir
A/Nagasaki/08N006/2009	2008–2009	10/01/2009	Oseltamivir
A/Niigata/08F031/2009	2008–2009	14/01/2009	Oseltamivir
A/Niigata/08F093/2009	2008–2009	19/01/2009	Oseltamivir
A/Niigata/08F188/2009	2008–2009	26/01/2009	Oseltamivir
A/Tottori/08T010/2008	2008–2009	31/12/2008	Oseltamivir

[a] NA, sensitive to both amantadine and oseltamivir.

formed full genome sequencing analysis of seasonal human influenza A/H1N1 viruses isolated in Japan during two influenza seasons, 2007–2008 and 2008–2009, to determine the genesis of antiviral drug-resistant viruses.

MATERIALS AND METHODS

Sample selection. Twenty-three clinical influenza A/H1N1 isolates obtained from different regions in Japan during the 2007–2008 and 2008–2009 seasons were selected to represent major phylogenetic clusters of the hemagglutinin (HA) and NA trees of previously analyzed viruses. These viruses were previously characterized and tested for their susceptibilities to the antiviral drugs amantadine and oseltamivir (2).

RNA extraction and full genome sequencing. RNA was extracted from 100 μl of the virus culture supernatants by using a commercial kit (Extragen II; Kainos, Japan), followed by reverse transcription with primer Uni12 (16) to make cDNA. PCR with primers with M13 overhangs on the 5′ ends was performed as described elsewhere (10). The PCR products were purified with an MSB Spin PCRapace kit (Invitek GmbH, Germany). Cycle sequencing was performed with a BigDye Terminator (version 3.1; Applied Biosystems) cycle sequencing kit, according to the manufacturer's instructions. The labeled products were then analyzed with an ABI Prism 3100 genetic analyzer (Applied Biosystems). The sequences were edited and assembled with SeqMan Pro software, included in the DNASTAR Lasergene package (Bioinformatics Pioneer DNASTAR, Inc.).

Phylogenetic analysis. Full genome sequences available for human influenza A/H1N1 viruses recovered from 2003 to 2008 were downloaded from the Influenza Virus Resource database (1). A total of 346 full genome sets were available, with sequences from 2007 constituting the majority (294 sets). A phylogenetic tree for each segment was constructed, followed by cluster analysis, which was performed with TreeDyn software (7), to determine the topology of each virus in the phylogenies of each of the eight segments (data not shown). On the basis of the preliminarily constructed phylogenies, a representative sample of 50 viruses from major branches was selected for inclusion in the final analysis, along with the 23 viruses sequenced in this study. The final data set included genes encoding polymerase basic protein 2 (PB2; 2,280 nucleotides [nt]), polymerase basic protein 1 (PB1; 2,274 nt), polymerase protein (PA) (2,151 nt), hemagglutinin (HA) (1,698 nt), nucleoprotein (NP) (1,497 nt), neuraminidase (NA) (1,413 nt), matrix protein (M) (982 nt), and nonstructural protein (NS) (838 nt). Sequence alignments were generated with BioEdit (version 7.0) software (12). The phylogenetic history was inferred for each individual segment by the neighbor-joining method with bootstrap analysis (n = 1,000) by use of the MEGA (version 4.0) program (36). A/New Caledonia/20/1999, the vaccine strain recommended by WHO for use in the 2000 to 2007 influenza seasons and for which the full genome was available from the Influenza Virus Resource database (1), was employed as an outgroup root for the trees. Clades were based on the HA tree, as described in the report of Hauge et al. (13).

Nucleotide sequence accession numbers. The genome sequences obtained in this study were deposited in the GenBank database under accession numbers CY043382 to CY043562.

RESULTS

The full genomes of the 23 influenza A/H1N1 isolates collected in Japan during the 2007–2008 and 2008–2009 influenza seasons were sequenced (Table 1). Five isolates from the 2007–2008 season were amantadine resistant and had the S31N mutation in the M2 protein, 6 isolates from the 2007–2008 season were amantadine and oseltamivir susceptible (antiviral drug susceptible), and 12 isolates from the 2007–2008 and 2008–2009 seasons were oseltamivir resistant and had the H275Y (N1 numbering) mutation. An additional 50 full genome sets for influenza viruses isolated in the United States, Australia, New Zealand, Japan, Nicaragua, and United Kingdom during the 2003 to 2008 seasons were included in the analysis; of the isolates in these samples, one isolate (recovered in the United States during the 2006–2007 season) was amantadine resistant and had the S31N mutation and one (recovered in the United Kingdom during the 2007–2008 season) was oseltamivir resistant and had the H275Y mutation.

The phylogenetic history of each of the genome segments is shown in Fig. 1. Viruses from 2006 to 2009 were assigned to two major clades, clades 1 and 2 (subclades 2A, 2B, and 2C), on the basis of the tree created with the HA gene and previously reported classifications (21). Clade 1 accommodated the majority of the viruses from the 2006–2007 season which possessed the antiviral drug-susceptible genotype, and the viruses

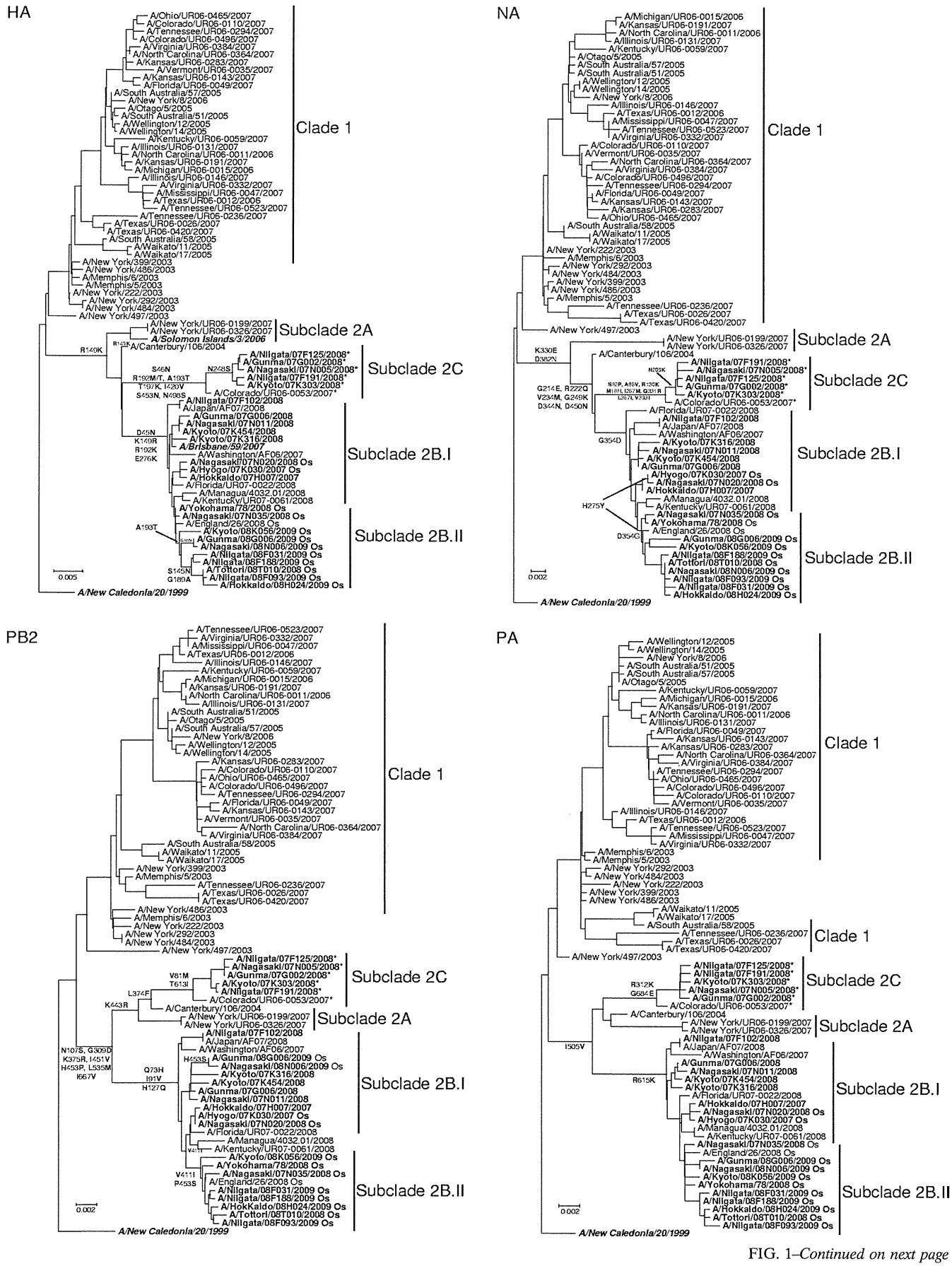
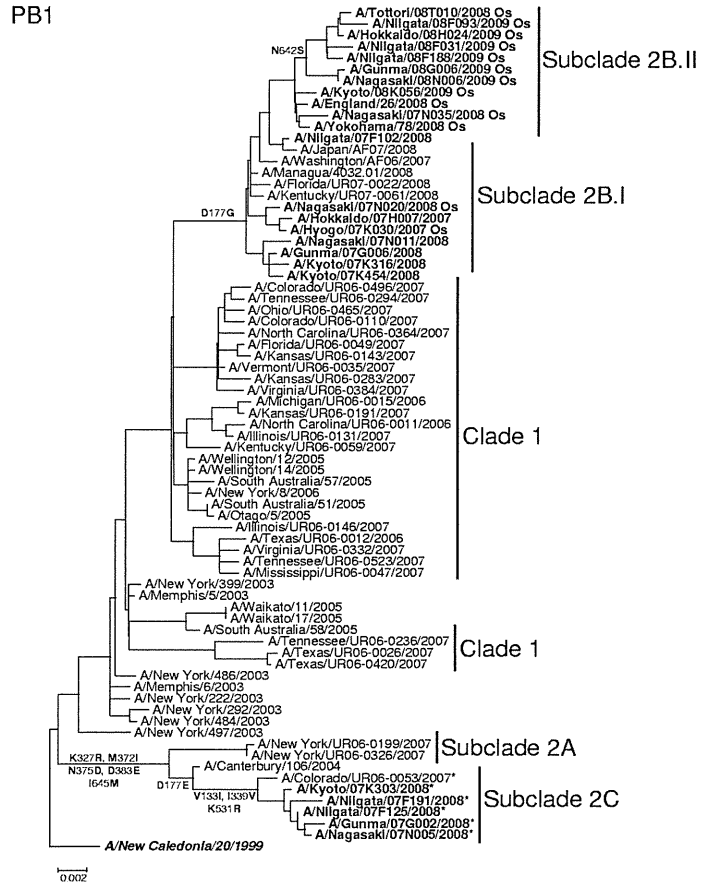
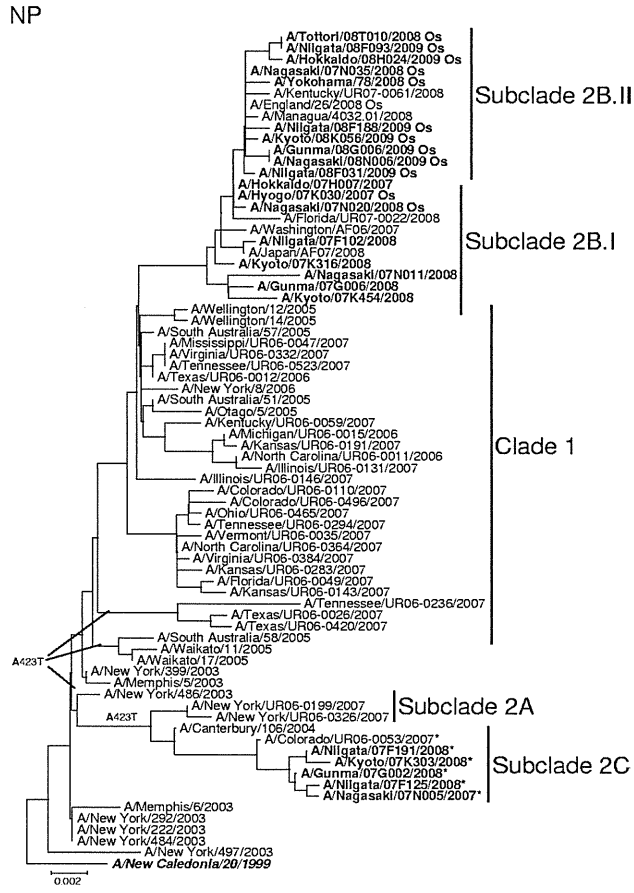
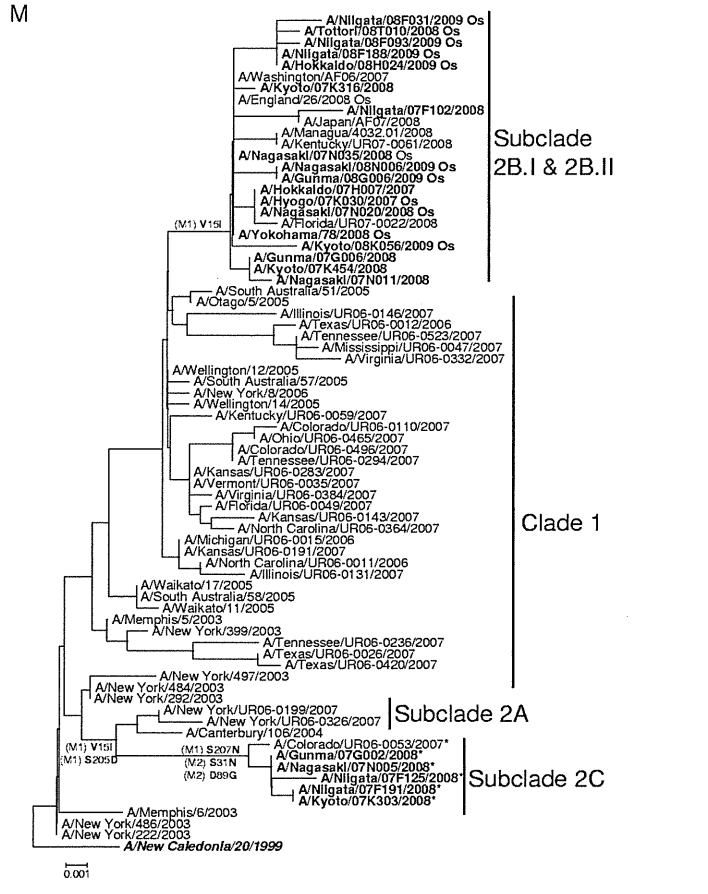
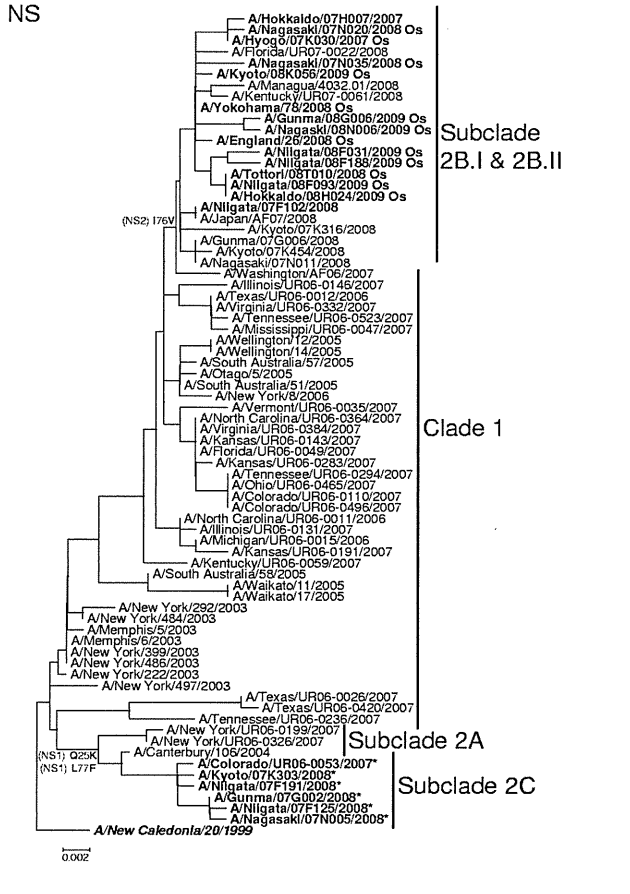


FIG. 1–*Continued on next page*









in clade 1 were most closely related to A/New Caledonia/20/1999-like virus (the vaccine strain recommended by WHO for use in the 2000 to 2007 seasons) in the HA phylogeny. Subclade 2A harbored antiviral drug-susceptible viruses from the 2006–2007 season, including A/Solomon Islands/3/2006-like virus (the vaccine strain for the 2007–2008 season). Subclade 2B could be further distinguished into two clusters, clusters 2B.I (representing the Hawaiian lineage) and 2B.II (representing the Northern Europe lineage). Subclade 2B.I harbored antiviral drug-susceptible viruses from the 2007–2008 season, including A/Brisbane/59/2007-like virus (the vaccine strain for the 2008–2009 season), in addition to two oseltamivir-resistant viruses with the H275Y mutation in their NA genes. Subclade 2B.II exclusively accommodated oseltamivir-resistant viruses from the 2007–2008 and 2008–2009 seasons that possessed the H275Y and D354G mutations in their NA genes. This lineage represented the major oseltamivir-resistant lineage that circulated in Europe during the 2007–2008 season (21) and in Japan and other countries during the 2008–2009 season. Subclade 2C accommodated amantadine-resistant viruses from the 2006–2007 and 2007–2008 seasons, and the viruses of subclade 2C were more closely related to A/Solomon Islands/3/2006-like viruses of subclade 2A.

In the phylogenies of all of the segments, amantadine-resistant subclade 2C was associated with the amantadine-susceptible A/Canterbury/106/2004 virus (the only virus from 2004 for which the full genome was available in the database) and was closely related to subclade 2A. On the other hand, subclade 2B had two different phylogenetic patterns. In the phylogenies of the PB2, PA, HA, and NA segments, subclade 2B was genetically related to subclades 2A and 2C, while in the phylogenies of the PB1, NP, M, and NS segments, it was most closely related to clade 1. Oseltamivir-resistant viruses, corresponding to the European resistant lineage, formed a distinct cluster (cluster 2B.II) within subclade 2B in the phylogenies of all segments except M and NS, in which oseltamivir-resistant and -susceptible viruses intermingled. This pattern strongly suggests that the oseltamivir-resistant strain has emerged in a reassortment event involving four segments each from a distinct oseltamivir-susceptible lineage, followed by the H275Y resistance-conferring mutation in the NA gene.

To gain better insight into the evolution of the amantadine-resistant A/H1N1 viruses and the oseltamivir-resistant A/H1N1 viruses, the amino acid sequence(s) of each segment was deduced. About 52 amino acid differences were found between subclade 2C amantadine-resistant viruses and subclade 2B amantadine-susceptible viruses (Fig. 1). These changes were not uniformly distributed among the 11 viral proteins. Of these mutations, 40 amino acid changes were detected in the subclade 2C background, and the remaining changes occurred in the subclade 2B background. The main oseltamivir-resistant strain (subclade 2B.II) had further mutations that consistently distinguished it from the oseltamivir-susceptible and -resistant viruses of subclades 2B.I and 2C: for PB2, the H453S mutation; for PB1, the N642S mutation (only viruses from the 2008–2009 season); for HA, the A193T mutation (only viruses from the 2008–2009 season); and for NA, the D354G mutation. The A193T mutation in HA (belongs to the receptor binding domain [RBD] and to the Sb antigenic site) was commonly detected among amantadine-resistant viruses and the major oseltamivir-resistant lineage, lineage 2B.II. All the A/H1N1 viruses sequenced in this study as well as those obtained from the database encoded for a truncated PB1-F2 protein of 57 amino acid residues.

DISCUSSION

Influenza A/H1N1 virus contributes to major epidemic strains and sometimes pandemic strains, such as the strain responsible for the current 2009 H1N1 pandemic. Genomic reassortment events between different strains of influenza A/H1N1 virus were associated with major antigenic variations and with the emergence of new strains (24, 25). Here we describe the results of a full genome sequence analysis of seasonal amantadine-resistant and oseltamivir-resistant influenza A/H1N1 viruses isolated during the 2007–2008 and 2008–2009 seasons in Japan. We show that amantadine-resistant A/H1N1 viruses originated from a strain that dates back to 2004, with extensive amino acid mutations occurring in the virus proteins. On the other hand, oseltamivir-resistant viruses were found to have evolved from a recent reassortant oseltamivir-susceptible strain during the 2007–2008 season by acquiring the H275Y resistance-conferring mutation in NA, in addition to minor amino acid modifications elsewhere in the genome.

The emergence of an amantadine-resistant influenza A/H3N2 virus strain worldwide in the 2003–2004 season has widely limited the utility of amantadine against influenza virus infections (3, 14, 28, 29). Despite that, the amantadine-resistant A/H1N1 virus strain still emerged worldwide during the following influenza season (8, 30). Our analysis revealed that this strain (subclade 2C) evolved independently of the major amantadine-susceptible strain (clade 1), predominantly detected in the 2006–2007 season, and that its closest genetic ancestry dates back to 2004.

In Japan, amantadine-resistant A/H1N1 viruses were first detected during the 2006–2007 season and were found to occur at a prevalence of 64.2% (30). They continued to circulate during the 2007–2008 season at a rate of 62.5% but virtually subsided and were replaced in the 2008–2009 season by the antigenically drifted amantadine-susceptible A/Brisbane/59/2007-like viruses (subclade 2B viruses in this study). The successful emergence and continued circulation of the amantadine-resistant A/H1N1 strain containing the S31N mutation in M2, despite the absence of amantadine usage in many countries, suggests an association of the S31N mutation with mu-

FIG. 1. Phylogenetic relationships of the genome segments of 74 influenza A/H1N1 virus isolates, including 23 genome sequences, determined in this study, of Japanese isolates (indicated in boldface) recovered between 2003 and 2009. All segment trees were rooted with the A/New Coledonia/20/1999 virus (the vaccine strain recommended by WHO for use during the 2001 to 2007 influenza seasons) for clarity. Amino acid mutations for key branches are shown. To determine the phylogenetic relationships of the HA genes of the viruses with recent vaccine strains A/Solomon Islands/3/2006 and A/Brisbane/59/2007, the vaccine strains for the 2007–2008 and 2008–2009 seasons, respectively, were employed (full genome sequences for these viruses were not available in the Influenza Virus Resource database). Vaccine strains are indicated in boldface italics. Asterisks, amantadine-resistant viruses; Os, oseltamivir-resistant viruses.

tations in other genome segments that favor its immune evasion or its replication. In this study, extensive mutations were observed in the genetic background of the amantadine-resistant viruses. The HA alone had 7 to 8 amino acid changes, 3 of which (R192M/T, A193T, and T197K) belonged to receptor binding and antigenic sites (4, 43). These changes might have altered the antigenicity of amantadine-resistant viruses and contributed to their ability to compete with susceptible ones. Nevertheless, this lineage was replaced by the emergence of an antigenically variant amantadine-susceptible A/Brisbane/59/2007-like strain, and a consistent drop in the prevalence of amantadine-resistant A/H1N1 during the 2008–2009 season was reported worldwide (5, 42).

On the other hand, the phylogenetic topology of subclade 2B, constituted of oseltamivir-susceptible and oseltamivir-resistant viruses, strongly suggested that the subclade 2B viruses are a product of a four segment plus four segment (PB2, PA, HA, NA + PB1, NP, M, NS) reassortment event involving two independent lineages. Subclade 2B accommodated A/Brisban/59/2007-like virus, a strain that has antigenically drifted from the A/Solomon Islands/3/2006 strain (subclade 2A), explaining the ability of the viruses of subclade 2B to prevail.

Oseltamivir-resistant A/H1N1 viruses emerged in the background of oseltamivir-susceptible subclade 2B viruses, as revealed by the close clustering of these viruses in the phylogenies of the eight segments, by acquiring the H275Y resistance-conferring mutation in NA. Neuraminidase inhibitors, including oseltamivir, were designed to closely resemble the natural sialic substrate, and thus, the notion was that NAI-resistant mutants would unlikely be able to retain normal enzyme activity (37). It was consistently shown that the viability and pathogenicity of A/H1N1 viruses with the H275Y mutation were severely compromised both *in vitro* and *in vivo* compared with the viability and pathogenicity of the corresponding wild type (15, 17), but contrasting data later emerged implying that the fitness of resistant viruses is determined instead by their genetic background (44). Furthermore, the recent emergence and rapid spread of the A/H1N1 viruses with the H275Y mutation in several regions of the world demonstrate that recent resistant viruses retain significant pathogenicity and transmissibility (9, 11, 13, 21, 40, 41). Data from *in vitro* analysis also show that oseltamivir-resistant viruses possess growth characteristics similar to those of oseltamivir-susceptible viruses and amantadine-resistant viruses (data not shown). Thus, at least *in vitro*, the drug resistance genotype does not affect the replication of these viruses. It is possible that oseltamivir-resistant viruses have benefited from the replication and transmissibility fitness available in the genetic elements of susceptible viruses and have further acquired slight genetic modifications that allowed them to predominate over the susceptible viruses. This should be further investigated in an animal model, namely, the ferret model, which would allow a better understanding of virus-host interactions, as well as the efficiency of transmissibility that might have contributed to the rapid increase in oseltamivir-resistant viruses, to be obtained.

In addition to the H275Y mutation, all oseltamivir-resistant viruses except two from the 2007–2008 season, had G354 in the NA gene, whereas susceptible viruses had a G354D mutation. The location of residue 354 at the top the neuraminidase tetramer and away from the enzyme binding site makes it unlikely that it compensates for the H275Y mutation (27). Interestingly, the main strain of oseltamivir-resistant viruses (clade 2B.II) which constituted the sole resistant strain in the 2008–2009 season in Japan and other countries (5, 42) acquired a mutation (A193T) in the 190 loop of the RBD of HA1 (43), in parallel with the H275Y resistance-conferring mutation in NA. Analysis of neuraminidase activity revealed that H275Y reduces the enzyme activity of N1 (44), which might have forced a parallel mutation in the RBD of the HA to retain a balance of activity.

The mutations observed in the polymerase complex (H453S in PB2 and N642S in PB1) of subclade 2B.II oseltamivir-resistant viruses might be also implicated in the improved overall fitness of these viruses in the human host. The former mutation (H453S in PB2) belongs to the nuclear localization signal of PB2 (amino acids 449 to 495), which binds to the α-importin (23). The latter mutation (N642S in PB1) is located within the PB2 binding site in PB1, which makes it interesting to study whether this mutation could compensate for the H127Q substitution, located in the PB1 binding site on the PB2 segment (20), that occurred in the background of subclade 2B viruses (both susceptible and resistant isolates).

It was recently suggested that the continued use of monotherapy should be reconsidered in favor of using a combination of an adamantane and a neuraminidase inhibitor (26). Such a strategy would be useful if widespread antiviral drug resistance had not yet occurred. However, now that resistance to the adamantanes and NAIs has already been established, the use of combined therapy might force the selection of viruses with double resistance. The cocirculation of amantadine-resistant and oseltamivir-resistant strains sets the ground for the mixing of these two lineages and, consequently, might result in a combination of the S31N resistance-conferring mutation in M2 and the H275Y resistance-conferring mutation in NA in one genetic background, as was recently reported in Hong Kong (6). Given the evolutionary nature of influenza virus, the possibility that such a strain might prevail is very likely. Rationalization of the usage of antivirals on the basis of epidemic type/subtype information in each area or limitation of the use of antivirals to severely compromised or high-risk patients, such as elderly individuals and children, until resistance subsides should be considered. On the other hand, the importance of vaccine selection should not be undermined, as a vaccine with a closer immunogenic match to the resistant strains would help eradicate resistant viruses and give a window for susceptible viruses to come back.

In conclusion, genomic modifications at the amino acid level and recombination events play important roles in producing transmissible antiviral-resistant influenza strains. Therefore, the continuation and strengthening of influenza surveillance programs and influenza genome projects so that they include viruses from undersurveyed areas are of great importance for the quick identification of such strains as they emerge.

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REFERENCES

1. **Bao, Y., P. Bolotov, D. Dernovoy, B. Kiryutin, L. Zaslavsky, T. Tatusova, J. Ostell, and D. Lipman.** 2008. The influenza virus resource at the National Center for Biotechnology Information. J. Virol. **82:**596–601.
2. **Baranovich, T., R. Saito, Y. Suzuki, H. Zaraket, C. Dapat, I. Caperig-Dapat, T. Oguma, I. I. Shabana, T. Saito, and H. Suzuki.** 2010. Emergence of H274Y oseltamivir-resistant A(H1N1) influenza viruses in Japan during the 2008–2009 season. J. Clin. Virol. **47:**23–28.
3. **Bright, R. A., M. J. Medina, X. Xu, G. Perez-Oronoz, T. R. Wallis, X. M. Davis, L. Povinelli, N. J. Cox, and A. I. Klimov.** 2005. Incidence of adamantane resistance among influenza A (H3N2) viruses isolated worldwide from 1994 to 2005: a cause for concern. Lancet **366:**1175–1181.
4. **Caton, A. J., G. G. Brownlee, J. W. Yewdell, and W. Gerhard.** 1982. The antigenic structure of the influenza virus A/PR/8/34 hemagglutinin (H1 subtype). Cell **31:**417–427.
5. **Centers for Disease Control and Prevention.** 2009. VRPBAC meeting briefing materials. Centers for Disease Control and Prevention, Atlanta, GA. http://www.fda.gov/ohrms/dockets/ac/09/briefing/2009-4416B1-1.pdf. Accessed 5 May 2009.
6. **Cheng, P. K., T. W. Leung, E. C. Ho, P. C. Leung, A. Y. Ng, M. Y. Lai, and W. W. Lim.** 2009. Oseltamivir- and amantadine-resistant influenza viruses A (H1N1). Emerg. Infect. Dis. **15:**966–968.
7. **Chevenet, F., C. Brun, A. L. Banuls, B. Jacq, and R. Christen.** 2006. TreeDyn: towards dynamic graphics and annotations for analyses of trees. BMC Bioinform. **7:**439.
8. **Deyde, V. M., X. Xu, R. A. Bright, M. Shaw, C. B. Smith, Y. Zhang, Y. Shu, L. V. Gubareva, N. J. Cox, and A. I. Klimov.** 2007. Surveillance of resistance to adamantanes among influenza A(H3N2) and A(H1N1) viruses isolated worldwide. J. Infect. Dis. **196:**249–257.
9. **Dharan, N. J., L. V. Gubareva, J. J. Meyer, M. Okomo-Adhiambo, R. C. McClinton, S. A. Marshall, K. St. George, S. Epperson, L. Brammer, A. I. Klimov, J. S. Bresee, and A. M. Fry.** 2009. Infections with oseltamivir-resistant influenza A(H1N1) virus in the United States. JAMA **301:**1034–1041.
10. **Ghedin, E., N. A. Sengamalay, M. Shumway, J. Zaborsky, T. Feldblyum, V. Subbu, D. J. Spiro, J. Sitz, H. Koo, P. Bolotov, D. Dernovoy, T. Tatusova, Y. Bao, K. St. George, J. Taylor, D. J. Lipman, C. M. Fraser, J. K. Taubenberger, and S. L. Salzberg.** 2005. Large-scale sequencing of human influenza reveals the dynamic nature of viral genome evolution. Nature **437:**1162–1166.
11. **Gooskens, J., M. Jonges, E. C. Claas, A. Meijer, P. J. van den Broek, and A. M. Kroes.** 2009. Morbidity and mortality associated with nosocomial transmission of oseltamivir-resistant influenza A(H1N1) virus. JAMA **301:**1042–1046.
12. **Hall, T. A.** 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. **41:**95–98.
13. **Hauge, S. H., S. Dudman, K. Borgen, A. Lackenby, and O. Hungnes.** 2009. Oseltamivir-resistant influenza viruses A (H1N1), Norway, 2007–08. Emerg. Infect. Dis. **15:**155–162.
14. **Hayden, F. G.** 2006. Antiviral resistance in influenza viruses—implications for management and pandemic response. N. Engl. J. Med. **354:**785–788.
15. **Herlocher, M. L., R. Truscon, S. Elias, H. L. Yen, N. A. Roberts, S. E. Ohmit, and A. S. Monto.** 2004. Influenza viruses resistant to the antiviral drug oseltamivir: transmission studies in ferrets. J. Infect. Dis. **190:**1627–1630.
16. **Hoffmann, E., J. Stech, Y. Guan, R. G. Webster, and D. R. Perez.** 2001. Universal primer set for the full-length amplification of all influenza A viruses. Arch. Virol. **146:**2275–2289.
17. **Ives, J. A., J. A. Carr, D. B. Mendel, C. Y. Tai, R. Lambkin, L. Kelly, J. S. Oxford, F. G. Hayden, and N. A. Roberts.** 2002. The H274Y mutation in the influenza A/H1N1 neuraminidase active site following oseltamivir phosphate treatment leave virus severely compromised both in vitro and in vivo. Antiviral Res. **55:**307–317.
18. **Kawakami, C., M. Obuchi, M. Saikusa, Y. Noguchi, M. Ujike, T. Odagiri, M. Iwata, T. Toyozawa, and M. Tashiro.** 2009. Isolation of oseltamivir-resistant influenza A/H1N1 virus of different origins in Yokohama City, Japan, during the 2007–2008 influenza season. Jpn. J. Infect. Dis. **62:**83–86.
19. **Kramarz, P., D. Monnet, A. Nicoll, C. Yilmaz, and B. Ciancio.** 2009. Use of oseltamivir in 12 European countries between 2002 and 2007—lack of association with the appearance of oseltamivir-resistant influenza A(H1N1) viruses. Euro. Surveill. **14**(5):pii=19112.
20. **Masunaga, K., K. Mizumoto, H. Kato, A. Ishihama, and T. Toyoda.** 1999. Molecular mapping of influenza virus RNA polymerase by site-specific antibodies. Virology **256:**130–141.
21. **Meijer, A., A. Lackenby, O. Hungnes, B. Lina, S. van-der-Werf, B. Schweiger, M. Opp, J. Paget, J. van-de-Kassteele, A. Hay, and M. Zambon.** 2009. Oseltamivir-resistant influenza virus A (H1N1), Europe, 2007–08 season. Emerg. Infect. Dis. **15:**552–560.
22. **Memoli, M. J., D. M. Morens, and J. K. Taubenberger.** 2008. Pandemic and seasonal influenza: therapeutic challenges. Drug Discov. Today **13:**590–595.
23. **Mukaigawa, J., and D. P. Nayak.** 1991. Two signals mediate nuclear localization of influenza virus (A/WSN/33) polymerase basic protein 2. J. Virol. **65:**245–253.
24. **Nelson, M. I., L. Edelman, D. J. Spiro, A. R. Boyne, J. Bera, R. Halpin, E. Ghedin, M. A. Miller, L. Simonsen, C. Viboud, and E. C. Holmes.** 2008. Molecular epidemiology of A/H3N2 and A/H1N1 influenza virus during a single epidemic season in the United States. PLoS Pathog. **4:**e1000133.
25. **Nelson, M. I., C. Viboud, L. Simonsen, R. T. Bennett, S. B. Griesemer, K. St. George, J. Taylor, D. J. Spiro, N. A. Sengamalay, E. Ghedin, J. K. Taubenberger, and E. C. Holmes.** 2008. Multiple reassortment events in the evolutionary history of H1N1 influenza A virus since 1918. PLoS Pathog. **4:**e1000012.
26. **Poland, G. A., R. M. Jacobson, and I. G. Ovsyannikova.** 2009. Influenza virus resistance to antiviral agents: a plea for rational use. Clin. Infect. Dis. **48:**1254–1256.
27. **Rameix-Welti, M. A., V. Enouf, F. Cuvelier, P. Jeannin, and S. van der Werf.** 2008. Enzymatic properties of the neuraminidase of seasonal H1N1 influenza viruses provide insights for the emergence of natural resistance to oseltamivir. PLoS Pathog. **4:**e1000103.
28. **Saito, R., D. Li, C. Shimomura, H. Masaki, M. Q. Le, H. L. Nguyen, H. T. Nguyen, T. V. Phan, T. T. Nguyen, M. Sato, Y. Suzuki, and H. Suzuki.** 2006. An off-seasonal amantadine-resistant H3N2 influenza outbreak in Japan. Tohoku J. Exp. Med. **210:**21–27.
29. **Saito, R., D. Li, Y. Suzuki, I. Sato, H. Masaki, H. Nishimura, T. Kawashima, Y. Shirahige, C. Shimomura, N. Asoh, S. Degawa, H. Ishikawa, M. Sato, Y. Shobugawa, and H. Suzuki.** 2007. High prevalence of amantadine-resistance influenza a (H3N2) in six prefectures, Japan, in the 2005–2006 season. J. Med. Virol. **79:**1569–1576.
30. **Saito, R., Y. Suzuki, D. Li, H. Zaraket, I. Sato, H. Masaki, T. Kawashima, S. Hibi, Y. Sano, Y. Shobugawa, T. Oguma, and H. Suzuki.** 2008. Increased incidence of adamantane-resistant influenza A(H1N1) and A(H3N2) viruses during the 2006–2007 influenza season in Japan. J. Infect. Dis. **197:**630–632.
31. **Salomon, R., and R. G. Webster.** 2009. The influenza virus enigma. Cell **136:**402–410.
32. **Simonsen, L., C. Viboud, B. T. Grenfell, J. Dushoff, L. Jennings, M. Smit, C. Macken, M. Hata, J. Gog, M. A. Miller, and E. C. Holmes.** 2007. The genesis and spread of reassortment human influenza A/H3N2 viruses conferring adamantane resistance. Mol. Biol. Evol. **24:**1811–1820.
33. **Stohr, K.** 2002. Influenza—WHO cares. Lancet Infect. Dis. **2:**517.
34. **Suzuki, Y., R. Saito, H. Zaraket, C. Dapat, I. Caperig-Dapat, and H. Suzuki.** 2010. Rapid and specific detection of amantadine-resistant influenza A viruses with a Ser31Asn mutation by the cycling probe method. J. Clin. Microbiol. **48:**57–63.
35. **Tamura, D., K. Mitamura, M. Yamazaki, M. Fujino, M. Nirasawa, K. Kimura, M. Kiso, H. Shimizu, C. Kawakami, S. Hiroi, K. Takahashi, M. Hata, H. Minagawa, Y. Kimura, S. Kaneda, S. Sugita, T. Horimoto, N. Sugaya, and Y. Kawaoka.** 2009. Oseltamivir-resistant influenza a viruses circulating in Japan. J. Clin. Microbiol. **47:**1424–1427.
36. **Tamura, K., J. Dudley, M. Nei, and S. Kumar.** 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol. Biol. Evol. **24:**1596–1599.
37. **Varghese, J. N., P. W. Smith, S. L. Sollis, T. J. Blick, A. Sahasrabudhe, J. L. McKimm-Breschkin, and P. M. Colman.** 1998. Drug design against a shifting target: a structural basis for resistance to inhibitors in a variant of influenza virus neuraminidase. Structure **6:**735–746.
38. **Weinstock, D. M., and G. Zuccotti.** 2006. Adamantane resistance in influenza A. JAMA **295:**934–936.
39. **Weinstock, D. M., and G. Zuccotti.** 2009. The evolution of influenza resistance and treatment. JAMA **301:**1066–1069.
40. **World Health Organization.** 2008. Influenza A(H1N1) virus resistance to oseltamivir—2008 influenza season, southern hemisphere. World Health Organization, Geneva, Switzerland. http://www.who.int/csr/disease/influenza/H1N1webupdate20082008_kf.pdf. Accessed 28 December 2008.
41. **World Health Organization.** 2009. Influenza A(H1N1) virus resistance to