(Masuda et al. 2000). First and nested PCR was performed to detect generic influenza A, using M2 gene primers (Masuda et al. 2000). Influenza B was detected in separate PCR runs using influenza B hemagglutinin gene primers (Shimizu et al. 1997). In this study, we defined "influenza infections" as PCR or virus isolation positive regardless of rapid test results (Fig. 1). This study was approved by the Medical Faculty Ethics Committee of the Niigata University, Graduate School of Medical and Dental Sciences.

Effectiveness of oseltamivir

Influenza-related fever was defined as body temperature of more than 37.5°C (99.5 F) using the highest body temperature among three different time measurements in a day. The effectiveness was evaluated by the fever duration more than 37.5°C after the first visit to clinic.

Statistical analysis

Statistical comparisons for baseline characteristics among the 4 groups by type of influenza and treatment were made by chi-square test to evaluate the proportions in multiple groups, and one-way analysis of variance to compare the mean values. Sheffe's test was used as univariate analysis to compare average values for the duration of fever among the four clinical groups. General linear model was employed as multi-variate analysis to assess independent variable which influenced the duration of fever and to estimate the adjusted average days for duration of fever by type and treatment. All statistical analyses were performed with SPSS 11.0J (SPSS Japan Inc., Tokyo). $P < 0.05$ was considered statistically significant.

Results

Patient characteristics

A total of 1,848 individuals with influenza-like illness were screened during the four successive seasons for the study (Fig. 1). Among these, 1,130 (61.1%) patients were positive for influenza with virus isolation or PCR, but nearly half of patients were excluded due to the reasons listed in the Fig. 1. As a result, a total of 602 patients (5 of

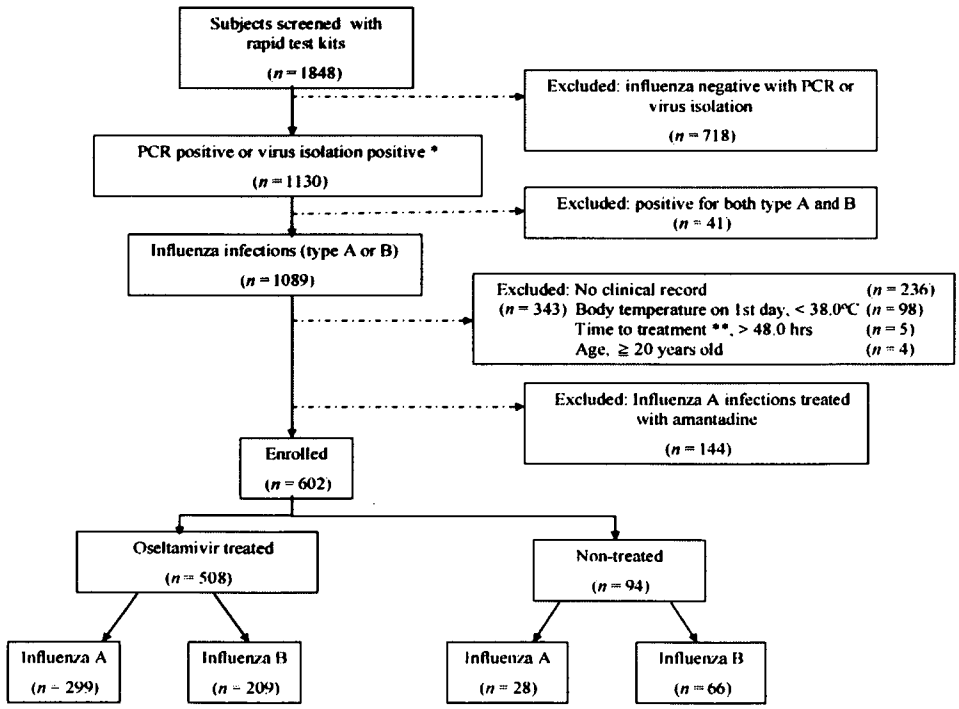

Fig. 1. Flow of participants through the study.
* Subjects were included regardless of rapid test results.
** Time until treatment, the time from the onset of fever to the first dose of treatment.

influenza A/H1N1, 257 of A/H3N2, and 257 of B were identified by virus isolation, and 65 of influenza A and 18 of influenza B by PCR) were enrolled in the study. They were divided into four groups by type of influenza and oseltamivir treatment status: 299 influenza A patients received oseltamivir treatment (treated influenza A), and 28 without treatment (non-treated influenza A), and 209 influenza B patients with treatment (treated influenza B) and 66 without treatment (non-treated influenza B), respectively (Table 1). The mean age and body weights, vaccination status, and the time until treatment did not differ significantly among the four groups. Body temperature at the time of clinic visit was higher in treated influenza A patients than treated influenza B, and younger patients (< 6 years old) had higher temperature than older ones (≧ 6 years old) in all groups.

Effectiveness of oseltamivir treatment for influenza A and B

The duration of fever was shorter in the treatment group as compared to the non treatment in influenza A (1.8 ± 0.9 days vs 2.6 ± 1.3 days; $p < 0.01$), but influenza B did not have statistical significance (2.4 ± 1.3 days vs 2.8 ± 1.2 days; $p = 0.09$) (Table 2). The fever duration was longer for influenza B treatment group (2.4 ± 1.3 days) than influenza A treatment group (1.8 ± 0.9 days; $p < 0.01$). In all four groups, duration of fever was significantly longer in younger (< 6 years old) than older children (≧ 6 years old) (Table 2). For younger group, the duration of fever was statistically shorter in treatment groups than non-treatment for both influenza A (3.1 ± 1.3 days vs 1.9 ± 1.0 days, $p < 0.01$, balance between the two = 1.2 days), and influenza B (3.2 ± 1.1 days vs 2.7 ± 1.3 days, $p < 0.05$, balance between the two = 0.5 days), but not in older children for both influenza

TABLE 1. Demographic details for influenza A and B patients by oseltamivir treatment.

	Influenza A		Influenza B		
	Oseltamivir non-treated ($n = 28$)	Oseltamivir treated ($n = 299$)	Oseltamivir non-treated ($n = 66$)	Osertamivir treated ($n = 209$)	p value[a]
Season					0.000
2001-2002	7 (25.0)	6 (2.0)	29 (43.9)	38 (18.2)	
2002-2003	6 (21.4)	28 (9.4)	0 (0.0)	6 (2.9)	
2003-2004	2 (7.1)	109 (36.5)	0 (0.0)	4 (1.9)	
2004-2005	13 (46.4)	156 (52.2)	37 (56.1)	161 (77.0)	
Gender					0.036
Male	9 (32.1)	175 (58.5)	39 (59.1)	109 (52.2)	
Female	19 (67.9)	124 (41.5)	27 (40.9)	100 (47.8)	
Age (years)	4.9 ± 4.0	5.8 ± 3.6	5.7 ± 2.3	6.4 ± 2.7	0.044
Body temperature at the clinic visit (°C)	39.1 ± 0.6	39.2 ± 0.6	38.9 ± 0.6	39.0 ± 0.6	0.000
Body weight (kg)	17.8 ± 10.0	22.5 ± 11.6	20.2 ± 6.1	22.4 ± 9.4	0.054
Time until treatment[b] (days)	1.0 ± 1.0	0.8 ± 0.6	0.8 ± 0.7	0.9 ± 0.7	0.208
Vaccination	7 (25.0)	112 (37.5)	17 (25.8)	83 (39.7)	0.115
Use of antifebrile drug	0 (0.0)	9 (3.0)	0 (0.0)	4 (1.9)	0.392

Numbers are mean ± S.D. or n (%).

[a] Chi-square test were employed for multiple rows and column contingency table, and one-way analysis of variance was used to compare means in multiple groups.

[b] Time until treatment, the time from the onset of fever to the clinic visit.

TABLE 2. Average duration of fever compared by uni-variate and multi-variate analysis by type of influenza and oseltamivir treatment.

	Uni-variate						Multi-variate					
	Influenza A			Influenza B			Influenza A patients			Influenza B patients		
	Non-treated	Oseltamivir treated	*p*	Non-treated	Oseltamivir treated	*p*	Non-treated	Oseltamivir treated	*p*	Non-treated	Oseltamivir treated	*p*
All age	2.6 ± 1.3 (n = 28)	1.8 ± 0.9 (n = 299)	< 0.01	2.8 ± 1.2 (n = 66)	2.4 ± 1.3 [a] (n = 209)	0.09	3.1 (2.5 - 3.6) [c] (n = 14)	2.0 (1.8 - 2.1) [c] (n = 228)	< 0.01	3.2 (2.9 - 3.5) [c] (n = 47)	2.8 (2.6 - 3.0) [a,c] (n = 176)	< 0.05
< 6 years	3.1 ± 1.3 (n = 18)	1.9 ± 1.0 (n = 158)	< 0.01	3.2 ± 1.1 (n = 33)	2.7 ± 1.3 [a] (n = 96)	< 0.05	3.6 (2.8 - 4.3) (n = 9)	2.1 (1.7 - 2.4) (n = 122)	< 0.01	3.5 (3.0 - 4.0) (n = 23)	2.9 (2.5 - 3.3) [a] (n = 80)	0.07
≧ 6 years	2.0 ± 1.2 [b] (n = 10)	1.6 ± 0.7 [b] (n = 141)	*n.s.*	2.5 ± 1.1 [b] (n = 33)	2.2 ± 1.3 [a,b] (n = 113)	*n.s.*	2.5 (1.6 - 3.4) (n = 5)	1.8 (1.6 - 2.1) (n = 106)	*n.s.*	2.9 (2.5 - 3.4) (n = 24)	2.6 (2.4 - 2.9) [a] (n = 96)	*n.s.*

Sheffe's test was used for uni-variate analysis, and general linear model was applied for multi-variate analysis for comparison of duration of fever ≧ 37.5°C after the first visit to the clinic.

Values indicate mean ± S.D. for uni-variate analysis, and mean with 95% confidence interval in brackets for multi-variate analysis, adjusted for age, sex, season, vaccination status, time until treatment, and the body temperature at the clinic visit.

n.s., not significant.

[a] Oseltamivir treated influenza A vs oseltamivir treated influenza B, $p < 0.01$.

[b] < 6 years vs ≧ 6 years for identical type and treatment group, $p < 0.05$.

[c] Those (n = 137) who were missing more than one of variables were excluded in multi-variate analyisis.

TABLE 3. Effects of influenza type, oseltamivir treatment, time until treatment, and maximum body temperature on the duration of fever analyzed with multi-variate analysis.

Factor	β (day)	p value
Influenza B virus infection	0.142	0.659
Oseltamivir treatment	−1.321	0.000
Age less than 6 years old	0.711	0.011
One degree higher body temperature at the clinic visit (°C)	0.550	0.000

General linear model was carried out with 465 patients, adjusted for gender, body weight, season, vaccination status and the time until treatment. Those (n = 137) who were missing more than one of variables were excluded from the analysis.

A (2.0 ± 1.2 days vs 1.6 ± 0.7 days, *n.s.*, balance between the two = 0.4 days), and influenza B (2.5 ± 1.1 days vs 2.2 ± 1.3 days, *n.s.*, balance between the two = 0.3 days). However, the fever duration was consistently shorter in treated influenza A than treated B for the two age categories.

We examined independent variable factors influencing the duration of fever using general linear model as multi-variate analysis (Table 3). Of variables analyzed, treatment of oseltamivir was a factor that attributed to the reduction of the fever duration by 1.32 days, whereas influenza B virus infection did not affect the illness duration significantly. Patients who were less than 6 years old exhibited the prolonged duration of fever by 0.71 days, and as well as one degree higher body temperature at the clinic visit by 0.55 days.

Average duration of fever was estimated in the four groups with adjustment for age, gender, body weight, influenza season, vaccination status, time until treatment, and body temperature at the clinic visit. The treatment groups had significantly shorter duration of fever than non-treatment groups for both influenza A (2.0 days vs 3.1 days, $p < 0.01$) and influenza B (2.8 days vs 3.2 days, $p < 0.05$) (Table 2). The duration was longer in treated influenza B than treated influenza A ($p < 0.01$), as in the uni-variate analysis. After stratification by age groups (< 6 years old, or ≧ 6 years old), average duration was consistently longer for all four groups in younger children than older ones (Table 2). In younger children (< 6 years old), the fever duration was significantly shorter in treated groups than non- treated for influenza A (3.6 days vs 2.1 days, $p < 0.01$, balance between the two = 1.5 days), but not for influenza B (3.5 days vs 2.9 days, $p = 0.74$, balance between the two = 0.6 days). In older children (≧ 6 years old), statistical significance was not demonstrated for both influenza A (2.5 days vs 1.8 days, *n.s.*, balance between the two = 0.7 days) and B (2.9 days vs 2.6 days, *n.s.*, balance between the two = 0.3 days). For the two age groups, treated influenza B had consistently longer fever duration than influenza A counterparts.

DISCUSSION

The clinical results in this paper provided evidence that oseltamivir was effective in reducing the duration of fever for both influenza A and B infections, but was less effective for influenza B infections rather than influenza A. Even after adjustment with various underlying factors, or categorization by age groups, the duration of fever in the treatment groups was consistently longer for influenza B than influenza A.

Oseltamivir has been thought to be equally effective against influenza A and B infections (Hayden et al. 1999; Whitley et al. 2001), but growing clinical evidence suggests oseltamivir is less effective against influenza B than influenza A. Our results were basically similar to the previous findings from Japan (Kawai et al. 2006; Sugaya et al. 2007). However, we emphasize that we carried out the study in multiple years, and enrolled sufficient number of non-treated groups for both influenza A and B in order to evaluate the effectiveness of the drug, in comparison with the pre-

vious studies implemented in a single year, and included relatively limited number of non-treated patients (Kawai et al. 2006; Sugaya et al. 2007). In addition, multi-variate analysis was employed to estimate the most influencing factors for the duration of fever, on the top of uni-variate analysis, which might be affected by confounding factors.

In our study, adjusted average duration of fever showed that treated influenza B had longer clinical course than treated influenza A. In vitro data suggested that the IC_{50} of influenza B virus to oseltamivir was higher than influenza A/H3N2 and A/H1N1 (Gubareva et al. 2001; Boivin and Goyette 2002; Hurt et al. 2004; Sugaya et al. 2007). Also, longer virus shedding was observed with influenza B than influenza A after oseltamivir treatment (Kawai et al. 2007). These data suggested that influenza B was less susceptible to oseltamivir than influenza A in vitro and in vivo. However, increasing the dosage for influenza B may not be advisable, since it prompts the issues of increased adverse effects. Choosing zanamivir for influenza B treatment is one of options (Kawai et al. 2008), but age limitations to this inhaling drug (≧ 5 years old) makes it difficult to generalize in pediatric practices.

Fever duration was consistently longer in younger children than older ones regardless of treatment. It is generally accepted that younger children with few previous influenza infections possessed prolonged course of illness and higher virus titer, due to insufficient inhibition of viral replication and higher cytokine levels. (Kiso et al. 2004; Kawai et al. 2008). In our analysis, oseltamivir seemed to be more effective in younger children than older children. It is true that insufficient number in some groups for older children (especially non-treated groups) made the results difficult to interpret, but statistical significance were more obvious in younger children than older ones. Also, the balance of fever duration between treated and non-treated was wider for younger children than older children for both influenza A and B. Despite the fact that the younger children had prolonged fever, the effect of treatment could be expected more in these groups. We need further investigations by enrolling larger number of children for confirmation.

In our multi-variate analysis, time from the onset to the clinic did not affect the fever duration as an independent variable. This is contrary to Kawai et al., reporting an increased benefit with early report to the clinic for the duration of fever (Kawai et al. 2008). These contrasting results were derived from the different criteria for fever duration between the two studies.

Higher body temperature at the first clinic visit was also a prolonging factor for fever duration as in the previous study (Kawai et al. 2008), suggesting the influences of higher viral replication and increased cytokines levels (Kiso et al. 2004; Kawai et al. 2008).

In conclusion, our study demonstrated the clinical effectiveness of oseltamivir for both influenza A and B patients, compared to non-treated patients. However, illness was prolonged for influenza B infections than influenza A under the treatment of oseltamivir.

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References

Aoki, F.Y., Macleod, M.D., Paggiaro, P., Carewicz, O., El Sawy, A., Wat, C., Griffiths, M., Waalberg, E. & Ward, P. (2003) Early administration of oral oseltamivir increases the benefits of influenza treatment. *J. Antimicrob. Chemother.*, **51**, 123-129.

Boivin, G. & Goyette, N. (2002) Susceptibility of recent Canadian influenza A and B virus isolates to different neuraminidase inhibitors. *Antiviral Res.*, **54**, 143-147.

Centers for Disease Control and Prevention (2006) Prevention

and Control of Influenza. Recommendations of the Advisory Committee on Immunization Practices (ACIP). *MMWR*, **55**, 24-30 (Also available at http://www.cdc.gov/mmwr/PDF/rr/rr5510.pdf).

Gubareva, L.V., Webster, R.G. & Hayden, F.G. (2001) Comparison of the activities of zanamivir, oseltamivir, and RWJ-270201 against clinical isolates of influenza virus and neuraminidase inhibitor-resistant variants. *Antimicrob. Agents Chemother.*, **45**, 3403-3408.

Hayden, F.G., Treanor, J.J., Fritz, R.S., Lobo, M., Betts, R.F., Miller, M., Kinnersley, N., Mills, R.G., Ward, P. & Straus, S.E. (1999) Use of the oral neuraminidase inhibitor oseltamivir in experimental human influenza: randomized controlled trials for prevention and treatment. *JAMA*, **282**, 1240-1246.

Hurt, A.C., McKimm-Breschkin, J.L., McDonald, M., Barr, I.G., Komadina, N. & Hampson, A.W. (2004) Identification of a human influenza type B strain with reduced sensitivity to neuraminidase inhibitor drugs. *Virus Res.*, **103**, 205-211.

Jefferson, T., Demicheli, V., Rivetti, D., Jones, M., Di Pietrantonj, C. & Rivetti, A. (2006) Antivirals for influenza in healthy adults: systematic review. *Lancet*, **367**, 303-313.

Kawai, N., Ikematsu, H., Iwaki, N., Satoh, I., Kawashima, T., Maeda, T., Miyachi, K., Hirotsu, N., Shigematsu, T. & Kashiwagi, S. (2005) Factors influencing the effectiveness of oseltamivir and amantadine for the treatment of influenza: a multicenter study from Japan of the 2002-2003 influenza season. *Clin. Infect. Dis.*, **40**, 1309-1316.

Kawai, N., Ikematsu, H., Iwaki, N., Maeda, T., Satoh, I., Hirotsu, N. & Kashiwagi, S. (2006) 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.*, **43**, 439-444.

Kawai, N., Ikematsu, H., Iwaki, N., Kawashima, T., Maeda, T., Mitsuoka, S., Kondou, K., Satoh, I., Miyachi, K., Yamaga, S., Shigematsu, T., Hirotsu, N. & Kashiwagi, S. (2007) Longer virus shedding in influenza B than in influenza A among outpatients treated with oseltamivir. *J. Infect.*, **55**, 267-272.

Kawai, N., Ikematsu, H., Iwaki, N., Maeda, T., Kanazawa, H., Kawashima, T., Tanaka, O., Yamauchi, S., Kawamura, K., Nagai, T., Horii, S., Hirotsu, N. & Kashiwagi, S. (2008) A comparison of the effectiveness of zanamivir and oseltamivir for the treatment of influenza A and B. *J. Infect.*, **56**, 51-57.

Kiso, M., Mitamura, K., Sakai-Tagawa, Y., Shiraishi, K., Kawakami, C., Kimura, K., Hayden, F.G., Sugaya, N. & Kawaoka, Y. (2004) Resistant influenza A viruses in children treated with oseltamivir: descriptive study. *Lancet*, **364**, 759-765.

Masuda, H., Suzuki, H., Oshitani, H., Saito, R., Kawasaki, S., Nishikawa, M. & Satoh, H. (2000) Incidence of amantadine-resistant influenza A viruses in sentinel surveillance sites and nursing homes in Niigata, Japan. *Microbiol. Immunol.*, **44**, 833-839.

Monto, A.S. (2003) The role of antivirals in the control of influenza. *Vaccine*, **21**, 1796-1800.

Moscona, A. (2005) Neuraminidase inhibitors for influenza. *N. Engl. J. Med.*, **353**, 1363-1373.

Mungall, B.A., Xu, X. & Klimov, A. (2004) Surveillance of influenza isolates for susceptibility to neuraminidase inhibitors during the 2000-2002 influenza seasons. *Virus Res.*, **103**, 195-197.

Nicholson, K.G., Aoki, F.Y., Osterhaus, A.D., Trottier, S., Carewicz, O., Mercier, C.H., Rode, A., Kinnersley, N. & Ward, P. (2000) Efficacy and safety of oseltamivir in treatment of acute influenza: a randomised controlled trial. Neuraminidase Inhibitor Flu Treatment Investigator Group. *Lancet*, **355**, 1845-1850.

Nicholson, K.G., Wood, J.M. & Zambon, M. (2003) Influenza. *Lancet*, **362**, 1733-1745.

Oxford, J.S., Bossuyt, S., Balasingam, S., Mann, A., Novelli, P. & Lambkin, R. (2003) Treatment of epidemic and pandemic influenza with neuraminidase and M2 proton channel inhibitors. *Clin. Microbiol. Infect.*, **9**, 1-14.

Oxford, J.S. (2005) Preparing for the first influenza pandemic of the 21st century. *Lancet Infect. Dis.*, **5**, 129-131.

Saito, R., Oshitani, H., Masuda, H. & Suzuki, H. (2002) Detection of amantadine-resistant influenza A virus strains in nursing homes by PCR-restriction fragment length polymorphism analysis with nasopharyngeal swabs. *J. Clin. Microbiol.*, **40**, 84-88.

Shimizu, H., Watanabe, S. & Imai, M. (1997) Rapid detection of influenza virus A (AH1, AH3) and B by nested-polymerase chain reaction. *Kansenshogaku Zasshi*, **71**, 522-526. (in Japanese)

Simonsen, L., Fukuda, K., Schonberger, L.B. & Cox, N.J. (2000) The impact of influenza epidemics on hospitalizations. *J. Infect. Dis.*, **181**, 831-837.

Sugaya, N., Mitamura, K., Yamazaki, M., Tamura, D., Ichikawa, M., Kimura, K., Kawakami, C., Kiso, M., Ito, M., Hatakeyama, S. & Kawaoka, Y. (2007) Lower clinical effectiveness of oseltamivir against influenza B contrasted with influenza A infection in children. *Clin. Infect. Dis.*, **44**, 197-202.

Treanor, J.J., Hayden, F.G., Vrooman, P.S., Barbarash, R., Bettis, R., Riff, D., Singh, S., Kinnersley, N., Ward, P. & Mills, R.G. (2000) Efficacy and safety of the oral neuraminidase inhibitor oseltamivir in treating acute influenza: a randomized controlled trial. US Oral Neuraminidase Study Group. *JAMA*, **283**, 1016-1024.

Whitley, R.J., Hayden, F.G., Reisinger, K.S., Young, N., Dutkowski, R., Ipe, D., Mills, R.G. & Ward, P. (2001) Oral oseltamivir treatment of influenza in children. *Pediatr. Infect. Dis. J.*, **20**, 127-133.

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Genetic Analysis of Influenza A/H3N2 and A/H1N1 Viruses Circulating in Vietnam from 2001 to 2006[▽]

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Influenza A virus has the ability to overcome immunity from previous infections through the acquisition of genetic changes. Thus, understanding the evolution of the viruses in humans is important for the surveillance and the selection of vaccine strains. A total of 30 influenza A/H3N2 viruses and 35 influenza A/H1N1 viruses that were collected in Vietnam from 2001 to 2006 were used to analyze the evolution of the hemagglutinin (HA), neuraminidase (NA), and matrix protein (M) genes. Phylogenetic analysis of individual gene segments revealed that the HA and the NA genes of the influenza A viruses evolved in a sequential way. However, the evolutionary pattern of the M gene proved to be nonlinear and was not linked with that of the HA and NA genes. Genetic drift in HA1 segments, especially in the antigenic sites of A/H3N2 viruses, occurred more frequently in A/H3N2 viruses than it did in A/H1N1 viruses. Two reassortants, one influenza A/H3N2 strain and one A/H1N1 strain, were found on the basis of the phylogenetic analysis of the three genes. While both genetic mutation and reassortment contributed to their evolution, the frequency of genetic changes and reassortment events differs between the two subtypes. As influenza viruses circulate throughout the year, we emphasize the importance of surveillance in tropical and subtropical zones, where the emergence of new strains may be detected earlier than it is in temperate zones.

The influenza virus is a major viral respiratory pathogen that causes yearly epidemics in tropical and subtropical countries, with epidemic influenza remaining a major cause of morbidity and mortality (27). Recurrent epidemics of influenza are due to the frequent emergence of antigenic variants. With the cocirculation of two influenza A subtypes, genetic reassortment also has an important role in antigenic drift (6, 28). The genome structure of influenza A viruses, consisting of eight segments of negative-sense single-stranded RNA, provides a basis for the remarkable antigenic variability in the human population through mutation and genetic reassortment. The influenza A virus surface glycoproteins, especially hemagglutinin (HA), are under selective pressure for change in order to evade the host's immune system. Thus, the HA and neuraminidase (NA) genes of influenza A viruses mutate at high frequencies (16, 17), resulting in the accumulation of point mutations that may lead to gradual antigenic changes in surface glycoproteins. This is known as antigenic drift. The matrix protein (M) gene, which encodes two viral proteins, M1 and M2, contributes to the control of virulence and growth (14, 36, 43, 44). Mutation in the M2 gene has been correlated with amantadine resistance (23).

The effectiveness of annually applied trivalent influenza vaccines depends on the selection of component strains that offer optimal immunity from the numerous variants in the global influenza virus circulation. Studies based on sequencing analyses of viruses can be utilized as surveillance tools and can contribute to the vaccine selection process when they are combined with classical serological antigenic analysis (10). Continuous monitoring of viral genetic changes throughout the year is necessary for us to develop our ability to precisely define variation in influenza virus.

In the tropical zone, influenza virus circulates throughout the year, as reported in southern China, Indonesia, and Thailand (2, 18, 20). A laboratory-based influenza virus surveillance system has been in place in Hanoi, Vietnam, since 2001, and we have reported on the presence of influenza virus throughout the year, albeit with summer and winter peaks (21). In order to elucidate the evolutionary patterns for influenza viruses in Vietnam, in this study we undertook a genetic analysis of the influenza A viruses circulating from 2001 to 2006, focusing on two external genes (the HA gene and the NA gene) and one internal regulatory gene (the M gene, which codes for the M1 and the M2 channel proteins).

MATERIALS AND METHODS

Virus collection and isolation. Nasopharyngeal swabs were obtained from outpatients residing in Hanoi and other provinces in Vietnam with symptoms of influenza-like illness from 2001 to 2006. The samples were placed in viral transport medium and transported to the Virology Department at the National Institute of Hygiene and Epidemiology on the day of collection. One hundred-microliter aliquots of the supernatants of the nasopharyngeal swabs were then inoculated onto Madin-Darby canine kidney cells, prepared in 48-well multiple-well plates. The plates were prepared at 37°C with 5% CO_2, and virus growth was monitored at 34°C with reference to cytopathic effects. The viruses were passaged three times to obtain sufficient virus titers for virus identification. All

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isolates were typed and subtyped by the hemagglutination inhibition assay (8). Selected virus isolates with sufficient titers were transferred for further analysis to the Department of Public Health, Niigata University Graduate School of Medical and Dental Science, for genetic analysis.

RNA extraction and PCR. One hundred-microliter aliquots of the supernatants after the third culture passage were used for viral RNA extraction with an Extragen II kit (Kainos, Tokyo, Japan), according to the manufacturer's instructions. RNA was transcribed to cDNA with the influenza A virus universal primer Uni12, as described elsewhere (24). The HA genes (segment 4), the NA genes (segment 6), and the M genes (segment 7) of H1N1 and H3N2 viruses were amplified with segment-specific primers as described elsewhere (3, 24).

Nucleotide sequencing and phylogenetic analysis. The PCR products were purified with a MicroSpin S-300 HR column PCR purification kit (Amersham Bioscience, Buckinghamshire, United Kingdom); labeled by use of a BigDye Terminator (version 3.1) cycle sequencing kit (Applied Biosystems, Foster, CA), according to the manufacturer's instructions; and then analyzed on an ABI 3100 automatic DNA sequencer. The sequences were assembled by use of the MEGA (version 3.1) program (26), and multiple-sequence alignment was conducted with the Clustal W program for the major coding regions of the three segments: HA1 (906 bp), NA (1,363 bp), and M (with regions of the overlapping reading frames of M1-M2; 923 bp) for H3N2 isolates and HA1 (829 bp), NA (1,363 bp), and M (938 bp) for H1N1 isolates. Phylogenetic trees were constructed by using a neighbor-joining and bootstrap analysis (n = 1,000) program to determine the best fits for the HA, NA, and M genes. Major branches with bootstrap values of >70% were identified as distinct groups. The phylogenetic grouping was not consistent for the three genes. The genomic sequences of the vaccine strains and the other strains used in this study were obtained from the Influenza Sequences Database (http://www.flu.lanl.gov).

Nucleotide sequence accession numbers. The nucleotide sequence data from this study were deposited in the DDBJ (DNA Data Bank of Japan), with the accession numbers listed in Table 1.

RESULTS

Thirty influenza A/H3N2 viruses that were collected from 2002 to 2005 and 35 influenza A/H1N1 viruses from 2001 to 2006 were used in this study. Samples were collected from various regions of Vietnam. No influenza A/H1N2 viruses were found during the study period.

A/H3N2 influenza virus. For analysis of the sequences of the HA, NA, and M genes of the influenza A/H3N2 viruses, we used the consensus sequence of A/Moscow/10/99 as the phylogenetic root for the HA gene. Other isolates were also used as reference strains for each year's epidemic.

In the phylogenetic tree of the HA genes of the A/H3N2 isolates, the viruses formed a monophyletic group which could be divided into four major subgroups with a bootstrap value of >70% (Fig. 1A). These four groups were concurrent with the influenza seasons: group I in 2002; group II in 2003; group III in 2003, 2004, and 2005; and group IV in 2005. Six isolates circulating during the 2002 season in Hanoi were in positions between the A/Panama/2007/99 and the A/Fujian/411/02 groups of viruses and were clustered in group I. One 2003 isolate and the vaccine strain (A/Fujian/411/02) were clustered in group II. Two isolates circulating in 2003, nine isolates circulating in 2004, and strain A/Wellington/1/04 were classified as group IIIa; and three isolates circulating in 2005 and strain A/California/7/04 were classified as group IIIb. The remaining nine isolates circulating in 2005 and the Northern Hemisphere vaccine strain A/Wisconsin/67/05 clustered in group IV.

Because the NA gene for strain Fujian/411/02 was not available from the database, one for a Fujian-like strain was chosen as a reference in the NA gene segment analysis. Analysis of the NA gene in this study showed that the viruses circulating from 2002 to 2005 were divided into two groups (Fig. 1B). Six 2002 isolates and one 2003 isolate clustered in group I (group I and group II for HA). The remaining isolates circulating in 2004 and 2005, an A/Fujian/411/02-like strain and strain A/Wiscon-

TABLE 1. Sequence data of influenza isolates used in this study

Strain group and strain	Collection date (yr.mo.day)	Accession no. of sequences used in this study[a]		
		Segment 4 HA gene	Segment 6 NA gene	Segment 7 MP gene
A/H3N2				
A/Hanoi/1872/02	2002.01.01	AB281205	AB281206	AB281207
A/Hanoi/184/02	2002.06.19	BAE75900	AB281193	AB281194
A/Hanoi/190/02	2002.06.23	AB281195	AB281196	AB281197
A/Hanoi/197/02	2002.06.26	BAE75901	AB281198	AB281199
A/Hanoi/209/02	2002.07.01	AB281200	AB281201	AB281202
A/Hanoi/235/02	2002.07.10	BAE75904	AB281203	AB281204
A/Hanoi/695/03	2003.08.14	AB221020	AB281208	AB281209
A/Tay Nguyen/TN152/03	2003.09.18	AB221034	NA[b]	NA
A/Tay Nguyen/TN160/03	2003.09.25	AB221035	NA	NA
A/Hanoi/HN3068/04	2004.01.18	AB281210	AB281211	AB281212
A/Hanoi/HN3069/04	2004.01.18	BAE75910	AB281213	AB281214
A/Hanoi/HN3094/04	2004.01.28	AB281215	AB281216	AB281217
A/Hanoi/HN30109/04	2004.01.30	BAE75911	AB281218	AB281219
A/Hanoi/HN30138/04	2004.02.04	AB281223	AB281224	AB281225
A/Hanoi/HN30135/04	2004.02.04	AB281220	AB281221	AB281222
A/Hanoi/ND049/04	2004.02.11	AB281229	AB281230	AB281231
A/Hanoi/HN30188/04	2004.02.12	AB281226	AB281227	AB281228
A/Hanoi/BG003/04	2004.02.13	AB284161	AB284162	AB284163
A/Hanoi/ISBM63/05	2005.05.09	AB281232	AB281233	AB281234
A/Hanoi/ISBM69/05	2005.05.09	AB281235	AB281236	AB281237
A/Hanoi/TB285/05	2005.05.18	AB281247	AB281248	AB281249
A/Hanoi/HN30607/05	2005.05.29	AB281241	AB281242	AB281243
A/Hanoi/HN30602/05	2005.05.30	AB281238	AB281239	AB281240
A/Hanoi/TN403/05	2005.10.11	AB281256	AB281257	AB281258
A/Hanoi/TN405/05	2005.10.13	AB281259	AB281260	AB281261
A/Hanoi/TN406/05	2005.10.13	AB281262	AB281263	AB281264
A/Hanoi/HN30720/05	2005.06.23	AB281244	AB281245	AB281246
A/Hanoi/TN388/05	2005.09.26	AB281250	AB281251	AB281252
A/Hanoi/TN410/05	2005.10.17	AB281265	AB281266	AB281267
A/Hanoi/TN399/05	2005.10.07	AB281253	AB281254	AB281255
A/H1N1				
A/Hanoi/1823/01	2001.12.21	AB285934	NA	AB285935
A/Hanoi/1863/01	2001.12.27	AB285936	AB285937	AB285938
A/Hanoi/1873/02	2002.01.01	AB285939	AB285940	AB285941
A/Hanoi/1892/02	2002.01.06	AB285942	NA	AB285943
A/Hanoi/1928/02	2002.01.14	AB285944	AB285945	AB285946
A/Hanoi/2006/02	2002.01.23	AB285947	AB285948	AB285949
A/Hanoi/188/02	2002.06.21	AB285950	NA	AB285951
A/Hanoi/191/02	2002.06.23	AB285952	AB285953	AB285954
A/Hanoi/337/03	2003.01.26	AB285955	NA	AB285956
A/Hanoi/784b/03	2003.06.25	AB285967	AB285968	AB285969
A/Hanoi/719/03	2003.08.26	AB285957	AB285958	AB285959
A/Hanoi/870b/03	2003.09.19	AB285970	NA	NA
A/Hanoi/777/03	2003.09.22	AB285960	AB285961	AB285962
A/Hanoi/893b/03	2003.10.10	AB285971	NA	NA
A/Hanoi/898b/03	2003.10.12	AB285972	NA	NA
A/Hanoi/902b/03	2003.10.16	AB285973	AB285974	AB285975
A/Hanoi/859/03	2003.10.24	AB285963	NA	AB285964
A/Hanoi/910b/03	2003.10.27	AB285976	NA	NA
A/Hanoi/867/03	2003.10.28	AB285965	NA	AB285966
A/Hanoi/979/03	2003.12.09	AB285981	NA	NA
A/Hanoi/981/03	2003.12.09	AB285982	NA	NA
A/Hanoi/949b/03	2003.12.10	AB285977	NA	NA
A/Hanoi/1004/03	2003.12.17	AB285983	AB285984	AB285985
A/Hanoi/1007/03	2003.12.18	AB285986	NA	NA
A/Hanoi/959b/03	2003.12.19	AB285978	AB285979	AB285980
A/Hanoi/1024/03	2003.12.24	AB285987	NA	NA
A/Hanoi/ISBM15/05	2005.03.24	AB285988	AB285989	AB285990
A/Hanoi/ISBM23/05	2005.04.01	AB285991	AB285992	AB285993
A/Hanoi/ISBM27/05	2005.04.05	AB285994	AB285995	AB285996
A/Hanoi/ISBM31/05	2005.04.08	AB285997	AB285998	AB285999
A/Hanoi/Q137/06	2006.03.07	AB286000	AB286001	AB286002
A/Hanoi/Q177/06	2006.03.15	AB286003	AB286004	AB286005
A/Hanoi/BM344/06	2006.05.11	AB286006	AB286007	AB286008
A/Hanoi/BM356/06	2006.05.19	AB286009	AB286010	AB286011
A/Hanoi/TX09/06	2006.06.08	AB286012	AB286013	AB286014

[a] All accession numbers are for the DNA Data Bank of Japan.
[b] NA, not addressed.

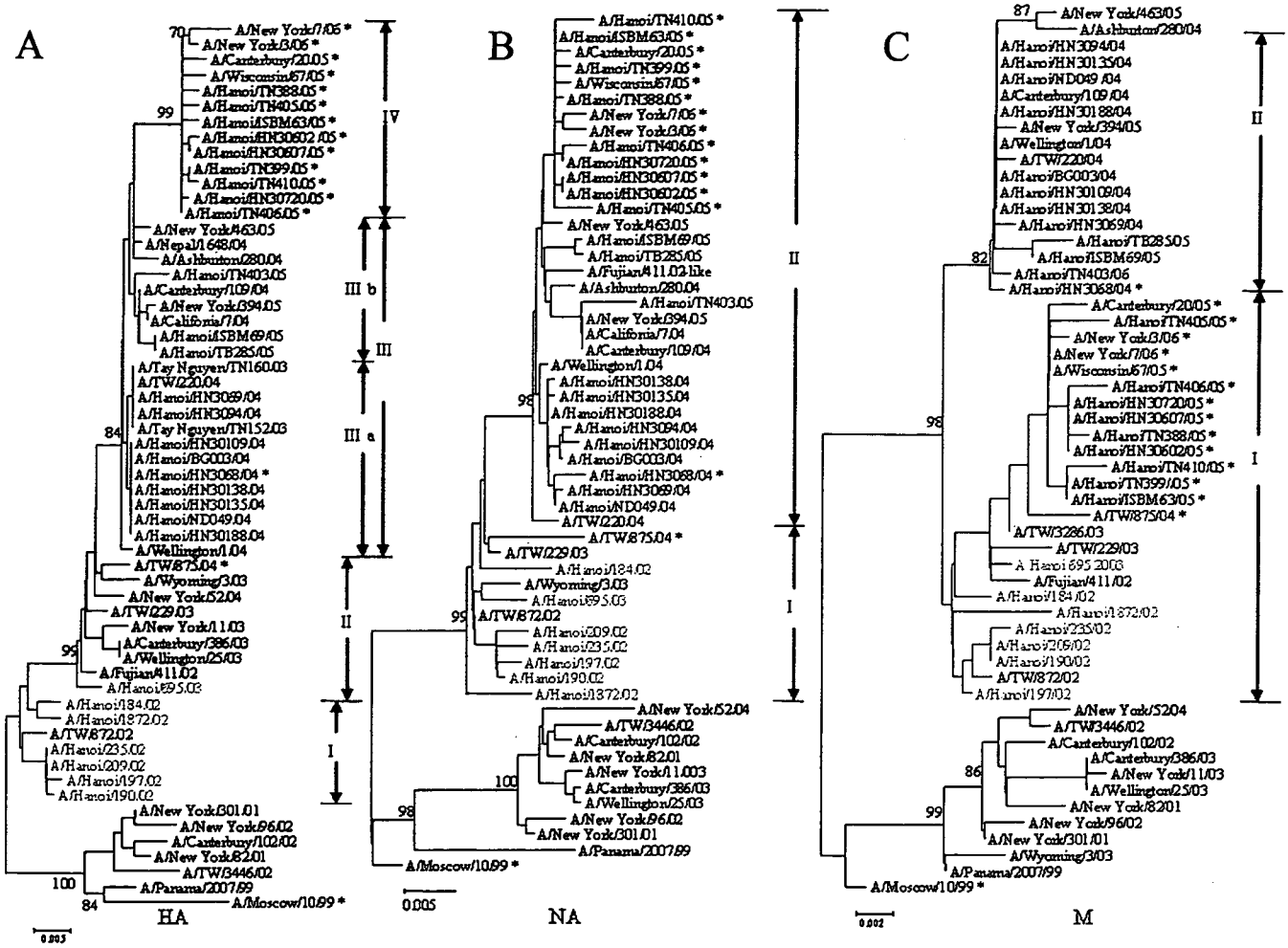

FIG. 1. Phylogenetic analysis of the HA1 domain of HA gene nucleotide sequences (906 bp), NA gene nucleotide sequences (1,363 bp), and M gene (with regions of the overlapping reading frames of M1-M2) nucleotide sequences (923 bp) of influenza A/H3N2 viruses circulating in Vietnam from 2002 to 2005. Reference strains shown in black were obtained from the genetic database. The isolates were assigned to group I (orange), group II (green), group III (purple), or group IV (red) in the phylogenetic tree of the HA gene (A). The grouping of the NA and the M genes was made in accordance with the branching in each tree. The colors of the strains in the phylogenetic trees of the NA gene (B) and the M gene (C) comply with those for the HA gene. Sequence data for the reference strains were obtained from the GenBank database. The asterisks denote the amantadine-resistant strain with the amino acid change S31N in the M2 protein. Bootstrap values of >70% are shown for the main groups.

sin/67/05, clustered in group II (group III and group IV for HA).

In the phylogenetic tree of the M gene (with regions of the overlapping reading frames of M1-M2), the viruses were divided into two groups. Isolates circulating in 2002, 2003, and 2005 were clustered in group I (group I, group II, and group IV for HA) (Fig. 1C). Nine isolates circulating in 2004 and three isolates circulating in 2005 were clustered in group II (group III for HA).

The amino acid sequences encoded by the HA (HA1 subunit) genes are shown in Table 2. For group I, all six isolates had amino acid changes, at positions S21P, R50G, E83K, N145K, S186G, V202I, W222R, and G225D, in comparison with the sequence of A/Panama/2007/99 strain. Four of the six isolates had additional double changes at positions N144D and G275D, and two of the six had an additional amino acid difference at position A131T. The single isolate circulating in 2003 and some other reference strains circulating in the same year were in group II and had four additional amino acid changes, L25I, H75Q, H155T, and Q156H, compared to the sequence of the isolates in group I. Among the isolates in group IIIa, 11 had additional changes at Y159F, S189N, and S227P and 4 of the 11 had another change at V226I, while among the isolates in group IIIb, three isolates circulating in 2005 further changed at K145N. All the remaining nine isolates in 2005, which were in group IV, had double amino acid changes at S193F and D225N. All isolates in this group were amantadine resistant and had the S31N amino acid change in the M2 gene.

A/H1N1 influenza virus. The phylogenetic tree for the HA genes of the A/H1N1 strains, including 35 isolates from 2001 to 2006 and 7 reference strains, showed four groupings (Fig. 2A). The isolates collected in 2004 were not included in our analysis because there was no collection of A/H1N1 viruses at the

TABLE 2. Comparison of amino acid sequences for the HA1 subunit of H3N2 viruses collected from 2002 to 2005

Group	Strain	Substitution at the following amino acid residue in HA1[a]:																			
		21	25	50[d]	75	83	131	144	145	155	156	159	186	189	193	202	222	225	226	227	275
	A/Panama/2007/99[b]	S	L	R	H	E	A	N	N	H	Q	Y	S	S	S	V	W	G	V	S	G
I	A/Hanoi/197/02	P	—[c]	G	—	K	—	D	K	—	—	—	G	—	—	I	R	D	—	—	D
I	A/Hanoi/1872/02	P	—	G	—	K	T	—	K	—	—	—	G	—	—	I	R	D	—	—	—
II	*A/Fujian/411/02*	P	I	G	Q	K	T	—	K	T	H	—	G	—	—	I	R	D	—	—	—
II	A/Hanoi/695/03	P	I	G	Q	K	T	—	K	T	H	—	G	—	—	I	R	D	—	—	—
IIIa	*A/Wellington/7/04*	P	I	G	Q	K	T	—	K	T	H	F	G	N	—	I	R	D	—	P	—
IIIa	A/Tay Nguyen/TN152/03	P	I	G	Q	K	T	—	K	T	H	F	G	N	—	I	R	D	—	P	—
IIIa	A/Hanoi/HN3069/04	P	I	G	Q	K	T	—	K	T	H	F	G	N	—	I	R	D	—	P	—
IIIa	A/Hanoi/HN3068/04	P	I	G	Q	K	T	—	K	T	H	F	G	N	—	I	R	D	I	P	—
IIIb	*A/Califonia/7/04*	P	I	G	Q	K	T	—	—	T	H	F	G	N	—	I	R	D	I	P	—
IIIb	A/Hanoi/ISBM69/05	P	I	G	Q	K	T	—	—	T	H	F	G	N	—	T	R	D	I	P	—
IV	*A/Wisconsin/67/05*	P	I	G	Q	K	T	—	—	T	H	F	G	N	F	I	R	N	I	P	—
IV	A/Hanoi/TN405/05	P	I	G	Q	K	T	—	—	T	H	F	G	N	F	I	R	N	I	P	—
IV	A/Hanoi/ISBM63/05	P	I	G	Q	K	T	—	—	T	H	F	G	N	F	I	R	N	I	P	—

[a] Results are reported as amino acid differences between the sequences of the isolates and the sequence of the A/Panama/2007/99 vaccine strain.
[b] Italic type indicates the reference vaccine strain.
[c] —, no change.
[d] Differences located in the proposed antigenic sites are underlined.

National Institute of Hygiene and Epidemiology. Among the samples, one isolate collected in 2001 and four isolates collected in 2002 were clustered in group I. The other 2 isolates from 2001 and 2002 and 18 isolates from 2003 clustered in group II. One isolate, A/Hanoi/1928/02, was localized between groups II and III. With the exception of one A/Hanoi/BM344/06 isolate, all samples from 2005 and 2006 clustered in group III. One A/Hanoi/BM344/06 isolate and the A/Solomon Islands/3/06 isolate, which is recommended as an influenza vaccine component in the 2007–2008 season in the Northern Hemisphere, were clustered in group IV, with a high bootstrap value of 100%.

Of the total 35 isolates, the NA genes of 20 isolates and the M genes of 26 isolates were examined. In the phylogenetic analysis of the NA genes, the isolates were divided into four groups (Fig. 2B). Intermediate strain A/Hanoi/1928/02 (in the position between groups II and III in the HA phylogenetic tree), two isolates from 2002, and one isolate from 2001 (group I for HA) were clustered in group I. Seven isolates from 2003 were clustered in group II (group II for HA). The isolates from 2005 and 2006 were divided into two groups: group III (group III for HA) and group IV. Isolate A/Hanoi/BM344/06 and isolate A/Hanoi/TX09/06, together with isolate A/Solomon Islands/3/06, which clustered in group III in the HA gene phylogenetic tree, were classified in group IV from the NA analysis.

In the phylogenetic analysis of the M genes (with regions of the overlapping reading frames of M1-M2) of the viruses, all except two isolates from 2001 to 2006 clustered in group I (Fig. 2C); isolates A/Hanoi/BM344/06 and A/Hanoi/TX09/06 clustered in group II with a bootstrap value of 89%.

In comparison to vaccine strain A/New Caledonia/20/99, the HA genes of the A/H1N1 isolates in groups I, II, and III from 2002 to 2006 had mutations at positions 158, 169, 190, 255, and 256. In particular, two amino acid changes at V169A and W255R consistently occurred in the samples (Table 3). At position 158, isolates in group II from 2002 and 2003 demonstrated an N158S change, while an isolate in group III from 2005 had a change at N158K. At position 190, isolates in groups I, II, and III demonstrated N190D and N190V changes. At position 256, isolates in group III from 2005 and 2006 featured Y256F. Isolate A/Hanoi/BM344/06 of group IV, on the other hand, exhibited 12 amino acid changes in comparison with the sequence of A/New Calidonia/20/99; these were E72D, K77R, T86K, Y98H, V132T, K144T, R149K, V169A, V190D, R212K, W255R, and T270N.

DISCUSSION

Influenza A viruses have segmented genomes consisting of eight RNA segments which encode viral proteins. Because of immunological pressure, genetic variability is mostly confined to regions of the genome responsible for viral surface proteins (33). However, other factors, including interactions of internal and surface proteins, are likely to affect viral fitness in a polygenic manner (25). Our present genetic analysis of A/H3N2 and A/H1N1 viruses isolated in Vietnam from 2001 to 2006 revealed various topologies for the phylogenetic trees of the HA and the NA genes, which encode two external proteins, and the M gene, which encodes an internal protein, indicating that genetic evolution did not occur at the same rate.

During the 5 years, influenza A/H3N2 virus evolved from a strain intermediate between A/Panama/2007/99 and A/Fujian/411/02 to an A/Wiscosin/67/05-like strain. The HA and NA genes evolved independently in a sequential way, whereas the M gene demonstrated a nonlinear pattern not linked to the evolution of the HA and NA genes (29). Furthermore, the HA gene could be divided into four groups with shifts from groups I to IV. The NA and M genes were divided into two groups, but the grouping of the M genes was not in accordance with that of the NA or the HA gene. The results also suggest that the HA gene evolved more rapidly than both the NA and the M genes and that these three genes of influenza A/H3N2 virus change in a nonsynchronous manner.

The genetic drift of the HA genes of the influenza A/H3N2 viruses, located at positions believed to have functional or

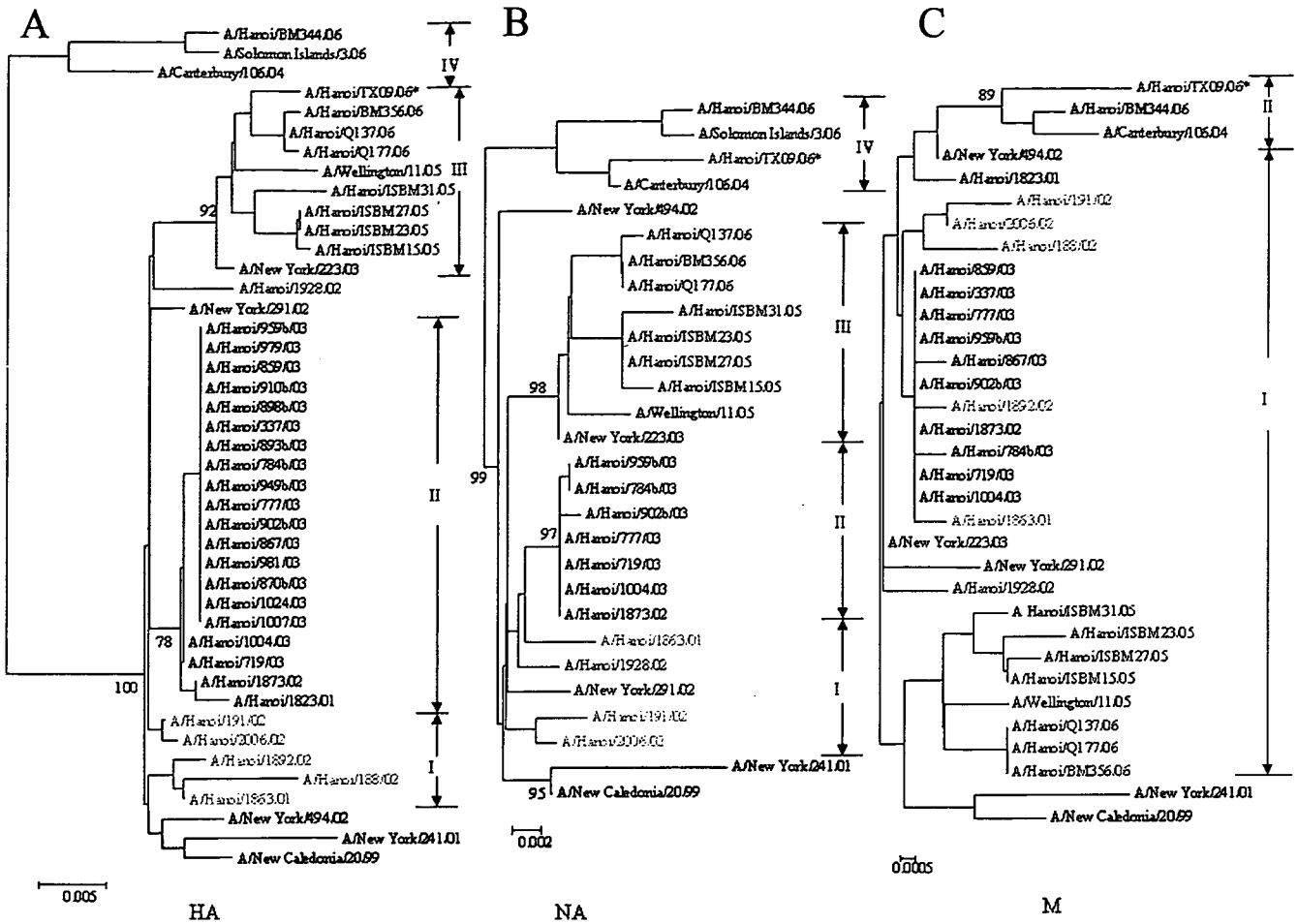

FIG. 2. Phylogenetic analysis of the HA1 domain of HA gene nucleotide sequences (829 bp), NA gene nucleotide sequences (1,363 bp), and M gene (with regions of the overlapping reading frames of M1-M2) nucleotide sequences (938 bp) of influenza A/H1N1 viruses circulating in Vietnam from 2001 to 2006. Reference strains shown in black were obtained from the genetic database. The isolates were assigned to group I (orange), group II (dark red), group III (purple), or group IV (red) in the phylogenetic tree of HA gene (Fig. 1A). The grouping of the NA and M genes was made in accordance with the branching in each tree. The colors of the strains in the phylogenetic trees of the NA gene (B) and the M gene (C) comply with those for the HA gene. The asterisks denote the amantadine-resistant strain with the amino acid change S31N in the M2 protein. Bootstrap values of >70% are shown for the main groups.

antigenic significance, was found to have occurred during all epidemic periods. The HA genes of viruses isolated in 2002 were clustered between strains A/Panama/2007/99 and A/Fujian/411/2002 in the phylogeny, with some strains having five amino acid changes within the antigenic sites (38, 39), namely, R50G (site C), E83K (site E), A131T (site A), N144K (site A), and S186G (site D). The same variant began to circulate in other parts of Asia between the end of 2001 and the beginning of 2002 (9). One isolate that was collected in August 2003 showed two key amino acid changes at residues H155T and Q156T (site B). This result indicated invasion of the A/Fujian/411/02 like strain into Vietnam, which was roughly coincident with our antigenic findings from influenza virus surveys in Hanoi (21). Between September 2003 and February 2004, the predominant strain circulating in Asia, the Americas, and Europe was an A/Fujian/411/02-like strain (40). However, two Vietnamese isolates circulating in September 2003 had drifted from the A/Fujian/411/02-like strain and showed additional changes at residues 159 (site B), 189 (site B), and 227 (site D). They had a higher degree of genetic homology to strain A/Wellington/1/04, which was isolated in New Zealand in January 2004 and which also circulated in Vietnam in the winter season of 2003–2004. Moreover, strain A/California/7/04 like, characterized by further changes at residues 145 (site A) and 226 (11, 30, 31), was isolated in Vietnam in May 2005 in this study.

High proportions of amantadine-resistant influenza A/H3N2 viruses with specific amino acid changes at residues 193 and 225 (named clade N) were reported from the 2005–2006 season in Japan and the United States (4, 12, 34, 35). Residue 193 is located within antigenic site B (37), and residue 225 is located within the receptor-binding site. In this study, clade N viruses were found from April to May of 2005 in Vietnam and the surrounding areas (1). However, it was in September and October of 2005 and the subsequent winter in Japan and North America when the strain caused a large community outbreak (5, 12, 34, 35).

The HA genes of the A/H1N1 isolates in groups I, II, and III

TABLE 3. Comparison of amino acid sequences for the HA1 subunit of H1N1 isolates collected from 2002 to 2006

Group	Strain	Substitution at the following amino acid residue in HA1 (H3 numbering)[a]:														
		77	81[d]	90	101	133	144	149	158	169	190	197	212	255	256	270
	A/New Caledonia/20/99[b]	E	K	T	Y	V	K	R	N	V	N	T	R	W	Y	T
I	A/Hanoi/1892/02	—[c]	—	—	—	—	—	—	—	A	D	—	—	R	—	—
I	A/Hanoi/1863/02	—	—	—	—	—	—	—	—	A	—	—	—	R	—	—
II	II A/Hanoi/1873/02	—	—	—	—	—	—	—	S	A	—	—	—	R	—	—
II	A/Hanoi/719/03	—	—	—	—	—	—	—	S	A	D	—	—	R	—	—
II	A/Hanoi/784b/03	—	—	—	—	—	—	—	S	A	V	—	—	R	—	—
III	III A/Hanoi/ISBM15/05	—	—	—	—	—	—	—	—	A	—	—	—	R	F	—
III	A/Hanoi/TX09/06	—	—	—	—	—	—	—	—	A	D	—	—	R	F	—
III	A/Hanoi/Q177/06	—	—	—	—	—	—	—	K	A	D	—	—	R	F	—
IV	IV A/Hanoi/BM344/06	D	R	K	H	T	E	K	—	A	D	—	K	R	—	N
IV	*A/Solomon Islands/3/06*	—	R	K	H	T	E	K	—	A	D	K	K	R	—	N

[a] Results are reported as amino acid differences between the sequences of the isolates and that of the A/New Caledonia/20/99 vaccine strain.
[b] Italic type indicates the reference vaccine strain.
[c] —, no change.
[d] Differences located in the proposed antigenic sites are underlined.

showed only five amino acid changes from the sequence of the A/New Caledonia/20/99 vaccine strain. The HA1 subunit includes the globular head and contains five major antibody-binding sites, namely, Sa, Sb, Ca1, Ca2, and Cb (7). A substitution at residue 169 (V169A) occurred in the Ca1 antigenic site in all isolates, while substitution at residue 158, located in the Sb site, changed irregularly with the strain. The WHO Influenza Center reported that the majority of H1N1 viruses circulating in 2005 were antigenically closely related to current vaccine strain A/New Caledonia/20/99 (22). One of the strains analyzed at the WHO center, A/Virginia/4/05 (22), is antigenically related to strain A/New Caledonia/20/99 and grouped together with our isolates in group III that were circulating in Vietnam in 2005 and 2006. Therefore, we conclude that the most of the H1N1 viruses circulated in Vietnam from 2001 to 2006 had antigenicity similar to that of strain A/New Caledonia/20/99 and underwent changes more slowly than the influenza A/H3N2 virus subtype, as reported elsewhere (15).

An A/Hanoi/BM344/06 strain collected in May 2006 showed 12 additional amino acid changes in the HA gene in comparison with the sequence of strain A/New Caledonia/20/99. Two of the 12 substitutions, K81R and V169A, were located in antigenic sites Cb and Ca1, respectively. In addition, substitutions at V133T and K144E were located in antibody-binding sites (38). Also, strain A/Solomon Islands/3/06, which was isolated in August 2006 and which was later recommended by WHO as the vaccine strain for the 2007–2008 season (41), clustered with strain A/Hanoi/BM344/06 in the phylogenetic tree of the HA gene. Therefore, it is likely that we obtained an early insight into the arrival of this novel strain of influenza A/H1N1 virus.

In general, reassortment events are reported more often in A/H3N2 viruses than in A/H1N1 viruses (25). Our analysis indicated that reassortment occurred for both H1N1 and H3N2 during this 6-year period. Isolate A/Hanoi/184/02 (H3N2) contained an HA gene intermediate between those of A/Panama/2007/99 and A/Fujian/411/02 and an NA gene similar to that of A/Wyoming/3/03, whereas the M gene proved similar to that of A/Fujian/411/02. For H1N1, isolate A/Hanoi/TX09/06 (H1N1) represents the result of a rarely reported reassortment. The HA gene of the virus was closely related to those in group III, which were antigenically related to the A/New Caledonia/20/99-like strain, but the NA and M genes were closely related to the A/Solomon Islands/3/06 strain. An intersubtype reassortant, A/H1N2, was isolated from Japan as well as from many other areas (13, 19, 32, 42) after 2001, but this was not detected in our present analysis.

Continuous monitoring of viral genetic changes throughout the year is warranted to monitor the variations of influenza viruses. As influenza viruses circulate throughout the year in the tropical and subtropical zones (18, 20), the results for viruses from those areas are very suitable for monitoring purposes. This applies especially in Asia, where the emergence of new strains may be detected earlier than the emergence of new strains in temperate zones, where there is only a single peak of activity in each year.

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REFERENCES

1. **Barr, I. G., A. C. Hurt, P. Iannello, C. Tomasov, N. Deed, and N. Komadina.** 2007. Increased amantadine resistance in influenza A (H3) viruses in Australia and neighboring countries in 2005. Antivir. Res. 73:112–117.
2. **Beckett, C. G., H. Kosasih, C. Ma'roef, E. Listiyaningsih, I. R. Q. Elyazar, S. Wuryadi, D. Yuwono, J. L. McArdle, A. L. Corwin, and K. R. Porter.** 2004. Influenza surveillance in Indonesia: 1999–2003. Clin. Infect. Dis. 39:443–449.

3. **Besselaar, T. G., L. Botha, J. M. McAnerney, and B. D. Schoub.** 2004. Antigenic and molecular analysis of influenza A (H3N2) virus strain isolated from a localized influenza outbreak in South Africa in 2003. J. Med. Virol. **73**:71–78.
4. **Bright, R. A., M. J. Medina, X. Xu, G. P. Oronoz, T. R. Wallis, X. M. Davis, L. Povinelli, N. 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.
5. **Bright, R. A., D. K. Shay, B. Shu, N. J. Cox, and A. I. Klimov.** 2006. Adamantane resistance among influenza A viruses isolated early during the 2005–2006 influenza season in the United States. JAMA **295**:891–894.
6. **Buonagurio, D. A., S. Nakada, J. D. Parvin, M. Krystal, P. Palese, and W. M. Fitch.** 1986. Evolution of human influenza A viruses over 50 years: rapid, uniform rate of change in NS gene. Science **232**:980–982.
7. **Caton, A., G. G. Brownlee, J. W. Yewell, and W. Gerhard.** 1982. The antigenic structure of the influenza virus A/PR/8/34 hemagglutinin (H1 subtype). Cell **31**:417–427.
8. **Center for Disease Control.** 1982. Concepts and procedures for laboratory based influenza surveillance. Center for Disease Control, Atlanta, GA.
9. **Chi, X. S., T. V. Bolar, P. Zhao, J. S. Tam, R. Rappaport, and S. M. Cheng.** 2005. Molecular evolution of human influenza A/H3N2 virus in Asia and Europe from 2001 to 2003. J. Clin. Microbiol. **43**:6130–6132.
10. **Cox, N. J., Z. S. Bai, and A. P. Kendal.** 1983. Laboratory-based surveillance of influenza A (H1N1) and A (H3N2) viruses in 1980–81: antigenic and genomic analyses. Bull. W. H. O. **61**:143–152.
11. **Daum, L. T., M. W. A. Shaw, I. Klimov, L. C. Canas, E. A. Macias, D. Niemeyer, J. P. Chambers, R. Renthal, S. K. Shrestha, R. P. Acharya, S. P. Huzdar, N. Rimal, K. S. Myint, and P. Gould.** 2005. Influenza A (H3N2) outbreak, Nepal. Emerg. Infect. Dis. **11**:1186–1191.
12. **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.
13. **Ellis, J. S., A. Alvarez-Aguero, V. Gregory, Y. P. Lin, A. Hay, and M. C. Zambon.** 2003. Influenza A (H1N2) viruses and their impact during the 2001–02 influenza season in the United Kingdom. Emerg. Infect. Dis. **9**:304–310.
14. **Enami, K., Y. Qiao, R. Fukuda, and M. Enami.** 1993. An influenza virus temperature-sensitive mutant defective in the nuclear-cytoplasmic transport of the negative-sense viral RNAs. Virology **194**:822–827.
15. **Ferguson, N. M., A. P. Galvani, and R. M. Bush.** 2003. Ecological and immunological determinants of influenza evolution. Nature **422**:428–433.
16. **Fitch, W. M., R. M. Bush, C. A. Bender, and N. J. Cox.** 1997. Long term trends in the evolution of H (3) HA1 human influenza type A. Proc. Natl. Acad. Sci. USA **94**:7712–7718.
17. **Fitch, W. M., R. M. Bush, C. A. Bender, K. Subbarao, and N. J. Cox.** 2000. Predicting the evolution of human influenza A. J. Hered. **91**:183–185.
18. **Gachara, G., J. Ngeranwa, J. M. Magana, J. M. Simwa, P. W. Wango, S. M. Lifumo, and E. O. Ochieng.** 2006. Influenza virus strains in Nairobi, Kenya. J. Clin. Virol. **35**:117–118.
19. **Gregory, V., M. Bennett, M. H. Orkhan, A. I. Hajjar, N. Varsano, E. Mendelson, M. Zambon, J. Ellis, A. Hay, and Y. P. Lin.** 2002. Emergence of influenza A H1N2 reassortant viruses in the human population during 2001. Virology **300**:1–7.
20. **Hampson, A. W.** 1999. Epidemiological data on influenza in Asian countries. Vaccine **17**(Suppl. 1):S19–S23.
21. **Hang, L. K. N., R. Saito, K. N. Ha, M. Nishikawa, Y. Shobugawa, C. N. Doan, T. H. Long, P. H. Lien, and H. Suzuki.** 2007. Epidemiology of influenza in Hanoi, Vietnam, from 2001 to 2003. J. Infect. **55**:58–63.
22. **Hay, A. J., and Y. Lin.** 2005. Characteristics of human influenza A H1N1, A H3N2 and B viruses isolated February to July 2005. WHO Influenza Centre, London, United Kingdom.
23. **Hay, A. J., A. J. Wolstenholme, J. J. Skehel, and M. H. Smith.** 1985. The molecular basis of the specific anti-influenza action of amantadine. EMBO J. **4**:3021–3024.
24. **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.
25. **Holmes, E. C., E. Ghedin, N. Miller, J. Taylor, Y. Bao, K. S. George, B. T. Grenfell, S. L. Salzberg, C. M. Fraser, D. J. Lipman, and J. K. Taubenberger.** 2005. Whole-genome analysis of human influenza A virus reveals multiple persistent lineages and reassortment among recent H3N2 viruses. PLoS Biol. **3**:1579–1588.
26. **Kumar, S., K. Tamura, and M. Nei.** 2004. MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief. Bioinform. **5**:150–163.
27. **Li, C. K., B. C. Choi, and T. W. Wong.** 2006. Influenza related deaths and hospitalizations in Hong Kong: a subtropical area. Public Health **120**:517–524.
28. **Lin, Y. P., V. Gregory, M. Bennett, and A. Hay.** 2004. Recent changes among human influenza viruses. Virus Res. **103**:47–52.
29. **Lindstrom, S. E., Y. Hiromoto, R. Nerome, K. Omoe, S. Sugita, Y. Yamazaki, T. Takahashi, and K. Nerome.** 1998. Phylogenetic analysis of the entire genome of influenza A (H3N2) viruses from Japan: evidence for genetic reassortment of the six internal genes. J. Virol. **72**:8021–8031.
30. **Macken, C., H. Lu, J. Goodman, and L. Boykin.** 2001. The value of a database in surveillance and vaccine selection, p. 103–106. *In* A. D. M. E. Osterhaus, N. Cox, and A. W. Hampson (ed.), Options for the control of influenza IV. Elsevier Science, Amsterdam, The Netherlands.
31. **Mar, A., R. Rahmanian, V. Lei, D. Lawrence, M. Krajden, R. C. Brunham, D. Skowronski, Y. Li, T. Booth, S. H. Goh, and M. Petric.** 2006. Longitudinal analysis of genotype distribution of influenza A virus from 2003 to 2005. J. Clin. Microbiol. **44**:3583–3588.
32. **Nishikawa, F., and T. Sugiyama.** 1983. Direct isolation of H1N2 recombinant virus from a throat swab of a patient simultaneously infected with H1N1 and H3N2 influenza A viruses. J. Clin. Microbiol. **18**:425–427.
33. **Reid, A. H., T. G. Fanning, T. A. Janczewski, and J. K. Taubenberger.** 2000. Characterization of the 1918 Spanish influenza virus neuraminidase gene. Proc. Natl. Acad. Sci. USA **97**:6785–6790.
34. **Saito, R., D. Li, C. Shimomura, H. Masaki, Q. E. Mai, L. K. N. Hang, T. N. Hien, T. N. Tien, V. P. Tu, T. K. N. Tien, M. Sato, Y. Suzuki, and Y. Suzuki.** 2006. An off-seasonal amantadine resistant H3N2 influenza outbreak in Japan. Tohoku J. Exp. Med. **210**:21–27.
35. **Saito, R., D. Li, and H. Suzuki.** 2007. Amantadine-resistant influenza A (H3N2) virus in Japan, 2005-2006. N. Engl. J. Med. **356**:312–313.
36. **Smeenk, C. A., and E. G. Brown.** 1994. The influenza virus variant A/FM/1/47-MA possesses single amino acid replacements in the hemagglutinin, controlling virulence, and in the matrix protein, controlling virulence as well as growth. J. Virol. **68**:530–534.
37. **Smith, D. J., A. S. Lapedes, J. C. de Jong, T. M. Bestebroer, G. F. Rimmelzwaan, A. D. Osterhaus, and R. A. Fouchier.** 2004. Mapping the antigenic and genetic evolution of influenza virus. Science **305**:371–376.
38. **Wiley, D. C., I. A. Wilson, and J. J. Skehel.** 1981. Structural identification of the antibody-binding sites of Hong Kong influenza hemagglutinin and their involvement in antigenic variation. Nature **289**:373–378.
39. **Wilson, I. A., and N. J. Cox.** 1990. Structural basis of immune recognition of influenza virus hemagglutinin. Annu. Rev. Immunol. **8**:737–771.
40. **World Health Organization.** 2004. Recommended composition of influenza virus vaccines for use in the 2004-2005 influenza season. Wkly. Epidemiol. Rec. **79**:88–92.
41. **World Health Organization.** 2007. Recommended composition of influenza virus vaccines for use in the 2007-2008 influenza season. World Health Organization, Geneva, Switzerland. http://www.who.int/csr/disease/influenza/vaccinerecommendations/en/index.html.
42. **Xu, X., C. B. Smith, B. A. Mungall, S. E. Lindstrom, H. E. Hall, K. Subbarao, N. J. Cox, and A. Klimov.** 2002. Intercontinental circulation of human influenza A (H1N2) reassortant viruses during the 2001-2002 influenza season. J. Infect. Dis. **186**:490–493.
43. **Yasuda, J., D. J. Bucher, and A. Ishihama.** 1994. Growth control of influenza A virus by M1 protein: analysis of transfectant viruses carrying the chimeric M gene. J. Virol. **68**:8141–8146.
44. **Yasuda, J., T. Toyoda, M. Nakayama, and A. Ishihama.** 1993. Regulatory effects of matrix protein variations on influenza virus growth. Arch. Virol. **133**:283–294.

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High Prevalence of Amantadine-Resistance Influenza A (H3N2) in Six Prefectures, Japan, in the 2005–2006 Season

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Substantial increase in amantadine-resistant influenza A (H3N2) was reported in Asia and North America in 2005. In this study the frequency and genetic characteristics of amantadine-resistant influenza A, circulated in Japan in 2005–2006 season, were investigated. Isolates were tested by amantadine susceptibility test ($TCID_{50}$/0.2 ml method), and sequencing of the M2 gene to identify mutations that confer resistance. Additionally, the hemagglutinin (HA) and neuraminidase (NA) genes of the viruses were examined. In total, 415 influenza A isolates from six prefectures were screened, and 231 (65.3%) of 354 influenza A (H3N2) were amantadine-resistant, with a serine to asparagine (S31N) change in the M2 gene. However, none of 61 A (H1N1) isolates were resistant. In addition, genetic analyses of the HA gene showed all amantadine-resistant viruses clustered in one (named clade N), possessing specific double mutations at 193, serine to phenylalanine (S193F), and at 225, asparatic acid to asparagine (D225N), and sensitive viruses belonged to another group (clade S). The clinical presentations at the clinical visit did not differ between patients shedding clade N virus and those shedding clade S virus. None of the patients had received previous treatment with amantadine. The results indicate an unusually high prevalence and wide circulation of the amantadine-resistance influenza A (H3N2) in Japan in the 2005–2006 season. These strains had the characteristic double mutations in the HA, in addition to the M2 mutation responsive for resistance. Antiviral resistance monitoring should be intensified and maintained for rapid feedback into treatment strategies, and selection of alternative therapeutic agents. ***J. Med. Virol. 79:1569–1576, 2007.*** © 2007 Wiley-Liss, Inc.

KEY WORDS: influenza; antiviral resistance; amantadine; genetic analysis

INTRODUCTION

Influenza is a main cause of acute respiratory infections during winter time, occurring annually in outbreaks of varying extent and severity. Antiviral therapy is available against influenza A viruses in the

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form of adamantanes (amantadine and rimantadine), and for both influenza A and B viruses, the neuraminidase (NA) inhibitors (oseltamivir and zanamivir) can be used [Dolin, 2005]. In Japan, amantadine has been licensed and prescribed by clinicians since 1998, but rimantadine has yet to be approved. NA inhibitors (oseltamivir and zanamivir) were licensed in 2001. Most clinicians in Japan use rapid diagnostic test kits for differential detection of influenza A or B in clinical settings, and select suitable drugs depending on the type of influenza [Kawai et al., 2005].

Amantadine is economical and chemically stable, but emergence of resistance and adverse effects are matters of concern [Suzuki et al., 2003]. The genetic basis for resistance is associated with amino acid substitutions at positions 26, 27, 30, and 31 in the transmembrane region of the M2 protein [Pinto et al., 1992; Holsinger et al., 1994; Pinto and Lamb, 2006]. Resistance after treatment may develop in approximately one third of patients [Hayden and Hay, 1992; Saito et al., 2002], but resistance in the pre-treatment samples, or community prevalence, has remained low in the past in Japan, at 0–5% [Suzuki et al., 2003; Kitahori et al., 2006], and less than 1% in the United States and the United Kingdom [Ziegler et al., 1999; Tooley, 2002]. However, Bright and colleagues recently highlighted a dramatic increase in the prevalence of amantadine-resistant A (H3N2) influenza strains in Asian countries and North America [Bright et al., 2005; Bright et al., 2006]. In Japan, an off-seasonal influenza A (H3N2) outbreak occurred in Nagasaki Prefecture in September 2005, which was highly linked to amantadine-resistant A (H3N2) circulation [Saito et al., 2006]. These resistant viruses belonged to a distinct genetic clade in A (H3N2) with double amino acid substitutions in the hemagglutinin (HA) gene, which had not been reported previously.

The objective of the present study was to clarify the prevalence of amantadine-resistance in six prefectures, Japan, in the 2005–2006 season, and to conduct a genetic analysis of lineages for resistant and sensitive A (H3N2) viruses.

MATERIALS AND METHODS

Sample Collections

Sixteen outpatient clinics and hospitals in six prefectures (four prefectures in the northeastern part of Japan, Miyagi, Yamagata, Niigata, Gunma, and two in the southwestern part, Fukuoka, and Nagasaki) participated in this study from 11 November 2005 to 27 April 2006. As a rule, randomly selected influenza-like-illness (ILI) patients, who visited the medical facilities, were enrolled. Clinicians applied influenza rapid diagnostic test kits licensed in Japan, such as QuickVue Rapid SP influ (DS Pharma Biomedical Co., Ltd., Osaka, Japan), Espline Influenza A&B-N (Fujirebio Inc., Tokyo, Japan) and Quick S-Influ A/B "SEIKEN" (Denkaseiken Co., Ltd., Tokyo), and Capilia Flu A+B (Becton Dickinson Japan, Tokyo). After the written or oral-informed consent was obtained, nasopharyngeal swabs were collected from influenza A positive patients together with clinical information, such as sex, age, date and time of symptom onset and clinic visit, influenza vaccination history for the 2005–2006 season, recent overseas travel, and clinical manifestations at the time of report (fever, headache, cough, sputum, rhinorrhea, abdominal pain, vomiting, agitation, muscle or joint pain, or fatigue). In addition, medication used in the clinic for influenza (amantadine, oseltamivir, or zanamivir) was recorded. The swabs were washed in viral transport media and kept at 4°C until transportation to laboratories for virus isolation within 1 week. The samples collected from Niigata, Nagasaki, and Gunma underwent virus isolation at the Department of Public Health, Niigata University Graduate School of Medical and Dental Sciences, Niigata City, Japan, and those from Miyagi, Yamagata and Fukuoka underwent isolation at Virus Research Center, Sendai Medical Center Hospital, Sendai City. All antiviral susceptibility testing and the genetic analysis were conducted in Niigata University.

Virus Isolation and Antiviral Susceptibility Testing

One hundred μl aliquots of supernatants of nasopharyngeal swabs were inoculated into Madin-Darby canine kidney cells (MDCK), prepared in 48-multiple well plates. The plates were kept at 34°C under at 5% CO_2 atmosphere for up to 10 days to assess cytopathic effects (CPE). Fifty μl aliquots of supernatants of CPE positive samples were then passaged twice to obtain a sufficient virus titer to perform virus identification and amantadine susceptibility testing. Influenza isolates were typed and subtyped by hemagglutination inhibition (HAI) assay with commercially available influenza vaccine strain antisera for the 2005–2006 season in Japan, A/New Caledonia/20/1999 (H1N1), A/New York/55/2004 (H3N2), B/Shanghai/361/2002 (Denka Seiken Co., Ltd., Tokyo, Japan), using guinea pig red blood cells.

$TCID_{50}$/0.2 ml, a previously reported phenotypic assay for amantadine susceptibility testing, was employed for all influenza A (H1N1) and A (H3N2) isolates [Masuda et al., 2000]. Two series of 10-fold dilutions of viruses from CPE-positive cultures were plated in triplicate in 96-well microplates with confluent MDCK cells, one dilution series containing a final concentration of 1.0 μg/ml of amantadine in the influenza virus maintenance media, and the other series being free of the drug [Masuda et al., 2000]. After incubation for 48 hr at 37°C, virus titers for amantadine-added and -free rows in triplicate were calculated by Reed–Muench format from the last dilution wells for which all cells are infected [Reed and Muench, 1938]. The susceptibility test was assessed as interpretable if the virus titer in amantadine-free rows exceeded 2.5 $\log_{10}$ $TCID_{50}$/0.2 ml. Amantadine-resistant strains were identified when less than two-fold difference in log $TCID_{50}$/0.2 ml titer was observed between series of rows with and without the drug.

Genetic Analysis

RNA was extracted from 100 μl of influenza isolates using an Extragen II kit (Kainos, Tokyo, Japan), according to the manufacturer's instructions. Reverse transcription (RT) to create complementary DNA (cDNA) was performed using influenza A generic primer, Uni12, as reported elsewhere [Hoffmann et al., 2001]. PCR was performed with the M2 gene-specific primers to amplify a 231-base pair product, covering nucleotides from 680 to 910 as described previously [Masuda et al., 2000]. The PCR product was visualized under UV light after electrophoresis with 3% agarose. The positive PCR products were sequenced to examine mutations at positions 26, 27, 30, or 31 in the transmembrane region of the M2 gene. The templates were labeled by cycle sequencing reactions with fluorescent dye terminators (BigDye Terminator v 3.1 Cycle Sequencing Kit, Applied Biosystems, Foster City, CA) and the products were sequenced using an ABI 3100 automatic sequencer (Applied Biosystems) following the manufacturer's instructions. Amplification and sequencing of the HA gene, segment 4, and the NA gene, segment 6, of influenza A (H3N2) were also performed with primers as reported elsewhere [Hoffmann et al., 2001; Besselaar et al., 2004].

A total of 86 concordant pairs of the HA and NA were obtained in this study. Three influenza A (H3N2) isolates collected in 2005 in National Institute of Hygiene and Epidemiology, Hanoi, and three isolates in Pasteur Institute in Ho Chi Minh City, previously reported as amantadine-resistant or -sensitive strains in Vietnam [Saito et al., 2006], were included in the analysis. In addition, sequence information for three of 2004–2005 isolates, collected in Niigata City in Japan (A/Niigata/F277/2005, A/Niigata/F757/2005, and A/Niigata/F759/2005), was involved to compare the genetic characteristics with 2005–2006 strains, together with recent influenza A (H3N2) strains registered in Influenza Sequence Database [Macken et al., 2001] and Influenza Virus Resource (http://www.ncbi.nlm.nih.gov/genomes/FLU/).

The nucleotide sequences of the HA and NA gene were aligned by using MEGA 3.1 software [Kumar et al., 2004]. Phylogenetic trees of the genes were constructed by using the neighbor-joining method and bootstrap analysis (n = 1,000) to determine the best-fitting tree for each gene [Saitou and Nei, 1987]. Bootstrap values ≥70% in major branches was assessed as distinct clades. The phylogenetic trees were drawn using MEGA 3.1 software.

Nucleotide Sequence Accession Numbers

The DNA Database of Japan (DDBJ, http://www.ddbj.nig.ac.jp/Welcome-e.html) accession numbers of the nucleotide sequences for the HA gene obtained in this study are AB270992–AB271002, AB271489–AB271498, AB271503–AB271506, AB271511–AB271517, AB271524–AB271530, and AB271809–AB271850. Those for the NA gene are AB271003–AB271006, AB271007–AB271009, AB271499–AB271502, AB271507–AB271510, AB271518–AB271523, AB271531–AB271537, and AB271809–AB271850.

Statistical Analysis

SPSS for Windows version 11.0 (SPSS Japan, Tokyo, Japan) was applied for the statistical analysis, for the Chi-squared test to compare rates in the two groups, and the Student's *t*-test to compare mean values. The level of statistical significance was set at 5%.

RESULTS

A total of 750 samples were collected during November 11, 2005–April 27, 2006 at 16 outpatient clinics in 6 prefectures in Japan before the start of any influenza treatment in the medical facilities (Table I). Among 415 influenza A strains isolated from those samples, 354 (85.3%) were for influenza A (H3N2) and 61 (14.7%) for A (H1N1) viruses.

All influenza A isolates underwent antiviral susceptibility testing and partial sequencing of the M2 gene. Two-hundred thirty one (65.3%) of 354 influenza A (H3N2) isolates had an amino acid substitution only at position 31, serine to asparagine (S31N) in the M2 gene that conferred amantadine-resistance, while none (0%) of 61 influenza A (H1N1) isolates showed any mutations in the region (Table I). The average age of cases demonstrating amantadine-resistance was 22.7 years, ranging from 0.2 to 106 years, and the proportion of males was 52.5%. The results of antiviral susceptibility assays were interpretable for 290 (69.9%) of 415 isolates. The sensitivity of the amantadine-susceptibility test compared to genetic sequencing was 91.7% (145/158), while the specificity was 86.1% (105/122).

The prevalence of amantadine-resistance A (H3N2) strains varied with the study sites (Table I), but all resistant strains had only the S31N mutation. The rates were 100% in Niigata, Gunma, and Yamagata prefectures. On the other hand, they were 68.2%, 36.8%, 42.2% in Miyagi, Fukuoka, and Nagasaki prefectures, respectively.

Demographic details and clinical manifestations of patients with amantadine-resistant and -sensitive A (H3N2) strains at the first clinic visit were compared with Nagasaki cases (Table II). In general, clinical presentations at the clinical visit did not differ between the two groups. All patients received anti influenza medication, mostly oseltamivir, and only small portion (around 1%) were treated with amantadine.

One patient infected with amantadine-resistant virus developed influenza symptoms 1 day after return from Korea to Nagasaki Prefecture at the end of December 2005 (A/Nagasaki/N07/2005). There was a family cluster of patients with resistant A (H3N2) virus in Gunma Prefecture; a 1-year-old baby with no prior medication infected with influenza, and her father developed symptoms 3 days later. Viruses collected from these two cases were amantadine-resistant A (H3N2) with the

TABLE I. Prefectures of Origin, the Rates of Amantadine Resistance by influenza A Subtypes, Median Age and Proportion of Sex of Patients Shedding Amantadine-Resistant A (H3N2)

Place[a]	Number of isolate/ sample[b]	Number of amantadine resistant/tested isolates				Am res age Mean (Range), years	Am res sex Male (%)
		H1N1	(%)	H3N2	(%)		
Miyagi	22/22	0/0	(0.0)	15/22	(68.2)	21.3 (1-74)	54.5
Yamagata	18/18	0/0	(0.0)	18/18	(100.0)	3.5 (0.4–8)	38.9
Niigata	152/426	0/51	(0.0)	101/101	(100.0)	5.4 (0.2–15)	52.6
Gunma	17/28	0/3	(0.0)	14/14	(100.0)	21.2 (1–58)	58.8
Fukuoka	20/20	0/1	(0.0)	7/19	(36.8)	8.6 (1–35)	45.0
Nagasaki	186/236	0/6	(0.0)	76/180	(42.2)	40.5 (5–106)	53.8
Total	415/750	0/61	(0.0)	231/354	(65.3)	22.7 (0.2–106)	52.5

Am res, patients shedding amantadine-resistant viruses.
[a]Place was sorted by the order of latitude for geographical location of prefectural government.
[b]Sample from Niigata, Gunma, and Nagasaki denotes nasopharygeal swabs, and those for Miyagi, Yamagata, and Fukuoka were virus isolates.

S31N mutation in the M2 gene, and had identical HA sequences (A/Gunma/K01/2006 and A/Gunma/K03/2006).

Phylogenetic tree analysis of the HA gene of influenza A (H3N2) viruses showed two different lineages, termed "clade N" [Saito et al., 2006] and "clade S" (Fig. 1). All of amantadine-resistant strains with the S31N mutation isolated from the six study sites belonged to clade N (clade N viruses), whereas all of amantadine-sensitive strains without M2 mutation isolated belonged to "clade S" (clade S viruses).

The HA_1 subunit in the HA gene (amino acid positions between 11 and 295) was analyzed to clarify the amino acid changes by strain. Two amino acid changes were found in clade N viruses, namely, serine 193 to phenylanalnine (S193F), and asparatic acid 225 to asparagine (D225N) (Table III). In clade S viruses, two amino acid changes were observed, namely, lysine 145 to serine (K145N), and asparatic acid 188 to tyrosine (D188Y).

On the other hand, the NA gene analysis showed clade N and S strains did not create independent

TABLE II. Demographic Details, Clinical Presentations at the First Clinic Visit, and Medication Against Amantadine Sensitive and Resistant A (H3N2) Cases

	Amantadine sensitive (N = 104)[a]	Amantadine resistant (N = 75)[a]	*P*-value[b]
Demographic details			
Age—year			
Average ± standard deviation	37.9 ± 17.5	44.7 ± 20.0	0.017
Range	8.3–83.8	6.8–83.0	
Male sex—number (%)	62 (59.6)	33 (44.0)	0.034
Time to clinic visit, days			
Average ± standard deviation	1.5 ± 1.1	1.5 ± 1.4	ns
Influenza vaccination—number (%)[c]	15 (14.4)	20 (26.7)	0.056
Recent travel to overseas—number (%)	0 (0.0)	1 (1.3)	ns
Clinical presentations—number (%)			
Fever, °C (average ± standard deviation)	38.4 ± 1.0	38.1 ± 0.9	0.041
Headache	71 (68.3)	53 (70.7)	ns
Cough	88 (84.6)	63 (84.0)	ns
Sputum	59 (56.7)	45 (60.0)	ns
Rhinorrhea	83 (79.8)	60 (80.0)	ns
Diarrhea	5 (4.8)	3 (4.0)	ns
Abdominal pain	6 (5.8)	6 (8.0)	ns
Vomiting	12 (11.5)	8 (10.7)	ns
Muscle or joint pain	74 (71.2)	50 (66.7)	ns
Fatigue	87 (83.7)	59 (78.7)	ns
Treatment—number (%)			
Oseltamivir	96 (92.3)	63 (84.0)	0.083
Zanamivir	7 (6.7)	11 (14.7)	ns
Amantadine	1 (1.0)	1 (1.3)	ns
Total[d]	104 (100.0)	75 (100.0)	

ns, not significant.
[a]Only cases in Nagasaki were included in the analysis.
[b]Statistical tests were employed by X^2 test to compare rates, and student's *t*-test to compare average values in pairs.
[c]History of influenza vaccination for 2005–2006 season.
[d]Number of cases who received any anti-influenza drug as treatment.

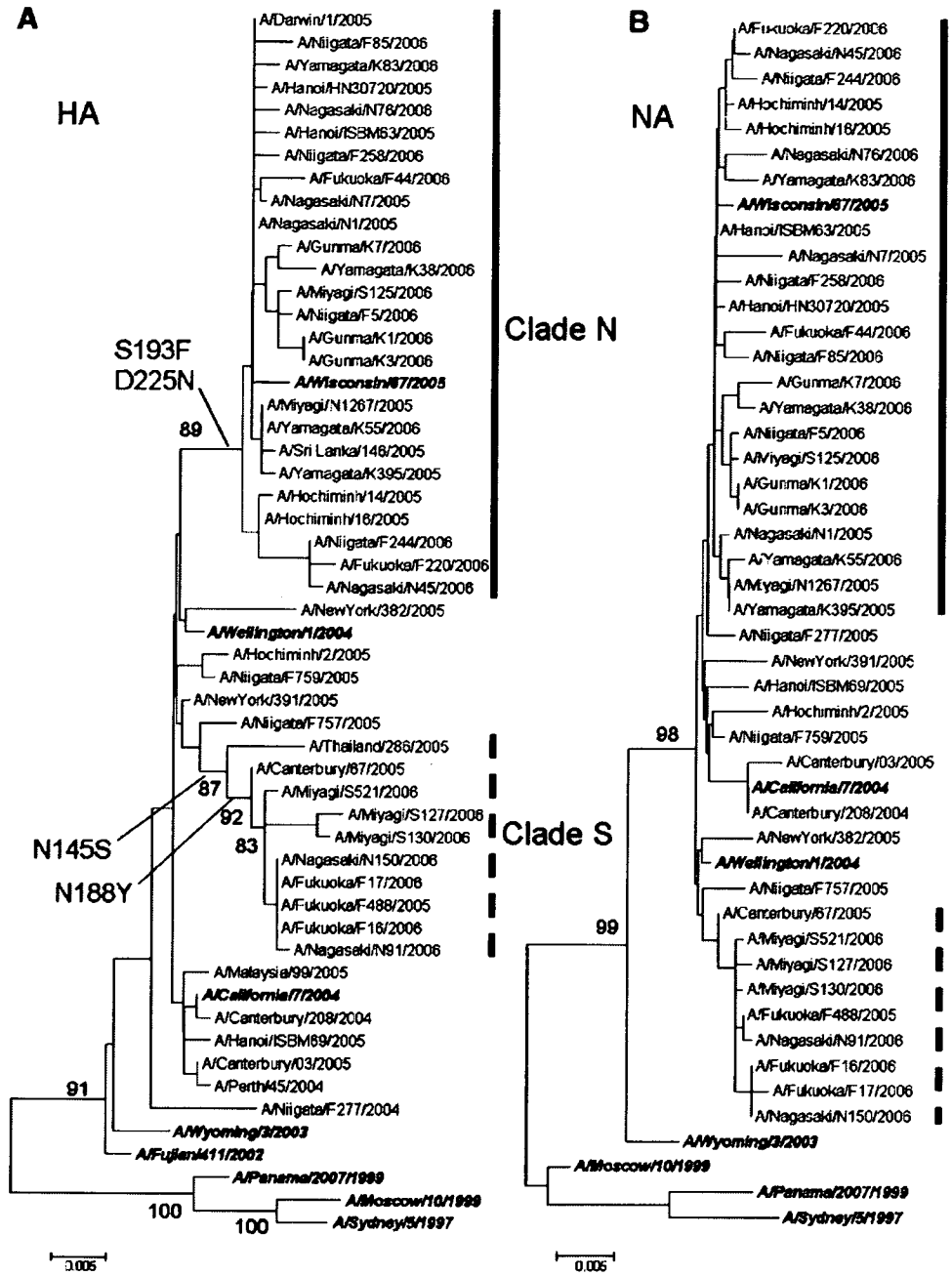

Fig. 1. Phylogenetic tree analysis among hemagglutinin (HA) genes (**Panel A**) and neuraminidase (NA) genes (**Panel B**) from human influenza A (H3N2) viruses sampled in Japan in 2005–2006 season. Only selected strains were shown in the figure. Virus names in italic boldface denote representative WHO-recommended influenza vaccine strains for A (H3N2) from 1999–2000 to 2006–2007 season. Phylogenetic trees were inferred from 853 nucleotide sequences for the HA (HA_1 subunit) and 1,388 nucleotide for the NA by the neighbor-joining method. Bootstrap values >70% in major branches are shown. The HA of influenza A (H3N2) strains carrying S31N mutation in the M2 gene, sampled in this study, formed a distinct cluster (named clade N, indicated by solid line) together with amantadine-resistant strains collected in Asian countries, the US, and New Zealand in 2005 (Panel A). This clade is characterized by possessing S193F and D225N mutations in the HA. Amantadine-sensitive strains clustered in one (named clade S, indicated by dashed line), carrying mutations N145S, N188Y in the HA. On the other hand, Panel B shows that in the NA, clade N and S strains did not form independent clusters, but were close to A/California/7/2004.

clusters as in the HA, but formed a common group together with the previous season strains, represented by A/California/7/2004 (vaccine strain for 2004–2005 season) (Fig. 1).

DISCUSSION

The emergence of resistance after treatment with adamantanes was an issue with their use, although

TABLE III. Amino Acid Differences Characteristic of A (H3N2) Hemagglutinin Between 2005–2006 and 2004–2005 Season Viruses

Variant group		Amantadine resistant		Amantadine sensitive			
		2004–2005	Clade N (2005–2006)	2004–2005	Clade S (2005–2006)		
Representative strain	A/California/7/2004[a]	A/Niigata/F277/2005[b]	A/Niigata/F206/2006	A/Niigata/F759/2005	A/Fukuoka/F20/2006	Functional significance	
H3 amino acid position							
145	N	N	N	N	S	Near 137[c]; receptor binding. Antigenic site A	
188	N	D	D	D	Y	Near 190[c]; receptor binding. Antigenic site B	
193	S	S	F	S	S	Near 194[c]; receptor binding. Antigenic site B	
225	D	D	N	D	D	Near 226[c]; receptor binding	

Am, amantadine.
[a]World Health Organization recommended influenza vaccine component for 2005–2006 season in Northern Hemisphere.
[b]The strain was primary amantadine resistant sampled in 2004–2005 in Niigata City, Japan.
[c]Amino acid residue contacts either sialic acid or penultimate galactose in the X31 virus HA complex with 3′-sialyllactose.

resistance usually appears only transiently, following treatment and had not resulted in significant spread or maintenance of resistant strains in circulation. The levels of community resistant viruses have been 0–5% in Japan and other part of the world [Ziegler et al., 1999; Suzuki et al., 2003; Kitahori et al., 2006]. However, a striking rise of community circulating amantadine-resistant influenza A (H3N2) was reported in China since 2003 [Bright et al., 2005], various Asian countries in 2005 [Saito et al., 2006; Barr et al., 2007b], and the United States and Canada in 2005–2006 season [Bright et al., 2006; Reyes et al., 2007]. Present study showed an unusually high prevalence (65.3%) and wide circulation of amantadine-resistant A (H3N2) viruses with the S31N mutation in Japan in the 2005–2006 season. These phenomena occurred despite the absence of sustained selective drug pressure and a decrease of total doses of amantadine administered, since none of amantadine-resistant patients in this report had a history of amantadine treatment before sample collection. Also, the estimated total number of amantadine-treated patients peaked in Japan in 2002–2003 for 2.7 million treatment courses (one treatment course = at a dosage of 100 mg for 5 days per person), then decreased steadily after the introduction of NA inhibitors, and only 0.1 million courses were given in 2005–2006 season.

The age distribution of patients shedding amantadine-resistant viruses was quite wide, and the majority were healthy residents. Also, clinical presentation at the first report did not differ between patients shedding amantadine-resistant and -sensitive viruses. According to the national influenza surveillance in Japan, the 2005–2006 epidemic was moderate in size, with average numbers of influenza patients per sentinel surveillance point in the peak week ranked as the 6th in the past 10 seasons (Infectious Disease Surveillance Center Japan, data available at http://idsc.nih.go.jp/idwr/kanja/weeklygraph/01flu-e.html). Therefore it was estimated that amantadine-resistant strains, circulated in the season, were on a similar level with sensitive ones as to virulence and transmissibility.

The phylogenetic tree analysis of the HA gene showed that all resistant strains circulated in this season belong to a single clade, clade N, whereas all their sensitive counterparts were clade S. In general, amantadine-resistance is attributed to the M2 gene mutation [Pinto and Lamb, 2006], and no apparent simultaneous changes were reported in other genes. In the case of subtype H7, some strains acquired amantadine-resistance through amino acid changes in the fusion protein (HA_2 subunit), but no change was found in the M2 [Steinhauer et al., 1991; Ilyushina et al., 2007]. This seems so far an exception related to subtype-specific protein functions [Ilyushina et al., 2007]. Thus, it was intriguing that all amantadine-resistant strains in this study had simultaneous changes in the HA, in addition to the M2 mutation.

The prevalence of clade N viruses in the 2005–2006 varied from 36.8% to 100% by area in Japan, while that of clade S was 0% to 63.2%. Viruses in each clade were

distributed in overlapping geographic regions. As shown by the genetic analysis, clade N viruses were also found in Vietnam [Saito et al., 2006], various areas in Asia [Barr et al., 2007b], the US (Influenza Virus Resource, available at http://www.ncbi.nlm.nih.gov/genomes/FLU/), and New Zealand (Influenza Virus Resource, available at http://www.ncbi.nlm.nih.gov/genomes/FLU/). One of the patients with a clade N virus (A/Nagasaki/N07/2005) developed influenza after travel to Korea, which strongly suggested circulation of the same amantadine-resistant strain in Korea. These observations led us to assume that clade N viruses predominated not only in Japan but also in Asia, North America, and Oceania during 2005–2006.

The conserved double mutations, S193F and D225N, for clade N viruses in the HA_1 subunit located in positions within a short distance of the site of receptor binding to cellular sialic acid, and those amino acid changes may affect antigenicity or transmissibility [Matrosovich et al., 2000; Suzuki et al., 2000; Medeiros et al., 2004]. Similar amino acid change was observed with the HA in clade I strains for highly pathogenic avian influenza A (H5N1), at position 189, which was relevant to 193 in A (H3N2). Clade I avian influenza circulated in Cambodia/Thailand/Vietnam in 2004–2005, and were all amantadine-resistant and highly transmissible [The World Health Organization Global Influenza Program Surveillance Network, 2005]. Thus, it was assumed that the high transmission fitness of these amantadine-resistant strains is associated with specific mutations in the HA. Therefore further study is needed to clarify the roles of the double mutations, S193F and D225N, alone or in combination, for the maintenance of S31N mutation in the M2.

The genetic grouping in the NA gene was not in accordance with that for the HA gene; the NA gene for both clade N and S strains grouped together with the strains circulated in 2004–2005, suggesting the NA gene did not undergo changes from the previous season, unlike with the HA. These findings implicated that the NA had little role to obtain the high transmission fitness for clade N viruses. Also, it was suggested that the frequency of genetic reassortment or mutations differed between the HA and NA, which was consistent with a recent report on whole genome analysis for A (H3N2) [Holmes et al., 2005].

Many of influenza A (H3N2) viruses in 2005–2006 in the world appeared closely related to the reference virus, A/Calfornia/7/2004, however, an increasing proportion was antigenically more closely related to A/Wisconsin/67/2005, and therefore WHO has recommended the strain as an influenza vaccine component for use in the 2006–2007 northern hemisphere influenza season [World Health Organization, 2006]. We found that this vaccine strain belonged to clade N as shown in the phylogenetic tree analysis, suggesting high possibility of linkage with amantadine-resistance. We also found a past vaccine component for 1999–2000, A/Moscow/10/99, carried S31N mutation in the M2 gene (Influenza Virus Resource, http://www.ncbi.nlm.nih.gov/genomes/FLU/). If it would be the case, amantadine-resistant viruses were also selected as a vaccine strain in the past. We need further study to clarify the influence to immunogenicity of vaccine and total yield of the HA protein with this resistant virus as vaccine component.

Japan is ranked as the top anti-influenza drug consumer in the world [Monto et al., 2006], and there is no restriction in the use of amantadine for the treatment and prophylaxis of influenza A infections. In this study, all patients received an anti-influenza drug, and the majority was treated with oseltamivir. The Advisory Committee on Immunization Practices in Centers for Disease Control and Prevention recommend that neither amantadine nor rimantadine is to be used for treatment or prophylaxis of influenza A in the United States for 2006–2007 season because of recent widespread occurrence of viruses resistant to these medications until susceptibility has been re-established among circulating influenza A viruses [Centers for Disease Control and Prevention, 2006]. However, seasonal influenza A strains as well as pandemic strains in the past, and currently circulating influenza A (H5N1) in Indonesia, China, and Mongolia mostly appear to be sensitive to amantadine [Hayden, 2006]. Furthermore, in our small-scale study, patients shedding primary amantadine-resistant viruses showed reduction in fever on amantadine treatment compared to emerged amantadine-resistance (unpublished work). Therefore, the actual clinical effectiveness of amantadine and NA inhibitors against amantadine-resistant strains needs to be evaluated in further studies.

In conclusion, high-level resistant human A (H3N2) viruses have emerged in Asia [Bright et al., 2006; Barr et al., 2007b]. No resistance was found for A (H1N1) in this study, but a recent report showed the prevalence of amantadine-resistance in A (H1N1) accounted for 21.8% in the samples collected in Oceania and South East Asia [Barr et al., 2007a]. It is essential to enhance systematic monitoring and evaluate clinical efficacy of antiviral treatment among patients with resistant viruses. The area of antiviral agents calls for maximum concentration of effort.

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