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Ⅳ. 研究成果の刊行物・別刷

15 FEBRUARY

Correspondence

Increased Incidence of Adamantane-Resistant Influenza A(H1N1) and A(H3N2) Viruses During the 2006–2007 Influenza Season in Japan

To the Editor—Deyde et al. [1] described an increased incidence of adamantane-resistant influenza A (H1N1) and A(H3N2) viruses worldwide during the 2005–2006 influenza season. Following their observations on the spread of adamantane resistance among A(H3N2) viruses in Asia and North America from 2001 to 2005 [2], we reported a similarly increased incidence of drug-resistant A(H3N2) viruses in Japan, which accounted for 65.3% of circulating strains during the 2005–2006 season [3]. Dual mutations, S193F and D225N, in the hemagglutinin (HA) molecule were noted in all resistant strains, in addition to the S31N mutation in the M2 gene that has conferred resistance (named clade N) [3]. Deyde et al. [1] call this group subclade 2b.

Here, we describe a follow-up study performed in Japan to assess the incidence of adamantane-resistant A(H1N1) and A(H3N2) viruses during the 2006–2007 season and to analyze genetic changes in the HA molecule. Between 22 November, 2006, and 18 May, 2007, we conducted an influenza study in 14 medical facilities located in 4 areas in Japan: Niigata, Gunma, Kyoto, and Nagasaki Prefectures. In total, 1453 nasopharyngeal swab samples were collected from patients with influenza-like illness who visited the different medical facilities. From these samples, 1004 influenza viruses were isolated; of these viruses, 120 (12.0%) were A(H1N1), 632 (62.9%) were A(H3N2), and 252 (25.1%) were B viruses.

The incidence of adamantane-resistant A(H1N1) viruses increased dramatically, rising from 0 of 61 (0%) during the 2005–2006 season to 77 of 120 (64.2%) during the 2006–2007 season; when evaluated by area, the incidence ranged from 52.4% to 100.0%. All resistant strains had a S31N mutation in the M2 gene. The viruses were genetically similar to A/Solomon Islands/3/2006, a new vaccine component for the 2007–2008 season, which is equivalent to what Deyde et al. [1] call clade 2 (figure 1) [1, 4]. Resistant and sensitive strains were categorized in different groups by genetic sequencing of the HA gene. All resistant strains had the amino acid changes R192M, A193T, and T197K, whereas sensitive strains had E276K. These 3 amino acid changes in resistant strains were localized near the receptor-binding and antigenic sites [4, 5]. However, these changes were not observed in the clade 2a strains from the 2005–2006 influenza season described in the report by Deyde et al. [1].

With respect to A(H3N2), 566 (89.6%) of 632 isolates were adamantane resistant and were shown to have the S31N mutation in the M2 gene by genetic sequencing [6]. The incidence of adamantane-resistant viruses was higher, compared with the past season (65.3% of viruses were resistant during the 2005–2006 season), and the percentage of resistant viruses in the 4 areas ranged from 80.6% to 94.8%. Genetic sequencing of the HA gene revealed that resistant A(H3N2) strains analyzed during the 2006–2007 season had amino acid changes S193F and D225N, as did viruses in clade N (i.e., subclade 2b) during the 2005–2006 season. Furthermore, sensitive strains also grouped together with resistant strains as clade N. (figure 1).

We confirmed an increased incidence of adamantane-resistant A (H3N2) and A (H1N1) virus strains during the 2006–2007 influenza season in Japan. At present, resistant strains circulate without selection pressure from the drug, because none of our patients was known to have received amantadine, as in other reports [7, 8]. The assumption was made that certain drastic genetic changes, such as substitutions in amino acid residues 193 and 225 in the HA molecule of clade N A(H3N2) virus, have occurred in the influenza genome that produce little or no fitness cost (i.e., reduction in transmissibility) [3, 9]. In particular, a change at position 193 may have significance because, in resistant strains, the same position was mutated in 3 subtypes, namely, resistant A(H1N1) in the 2006–2007 season, clade N virus with A(H3N2), and clade 1 viruses with A(H5N1) [10]. This specific HA mutation may contribute to the maintenance of adamantane-resistant strains.

Furthermore, after 2 seasons, it was observed that clade N A(H3N2) resistant strains accommodated sensitive strains that had dual mutations in the HA gene but lacked the M2 gene mutation (figure 1). This may indicate that the selection pressure still favors sensitive strains over resistant strains. With respect to recent human influenza, we need to elucidate the mechanism behind the appearance and maintenance of adamantane-resistant strains that acquired little fitness cost.

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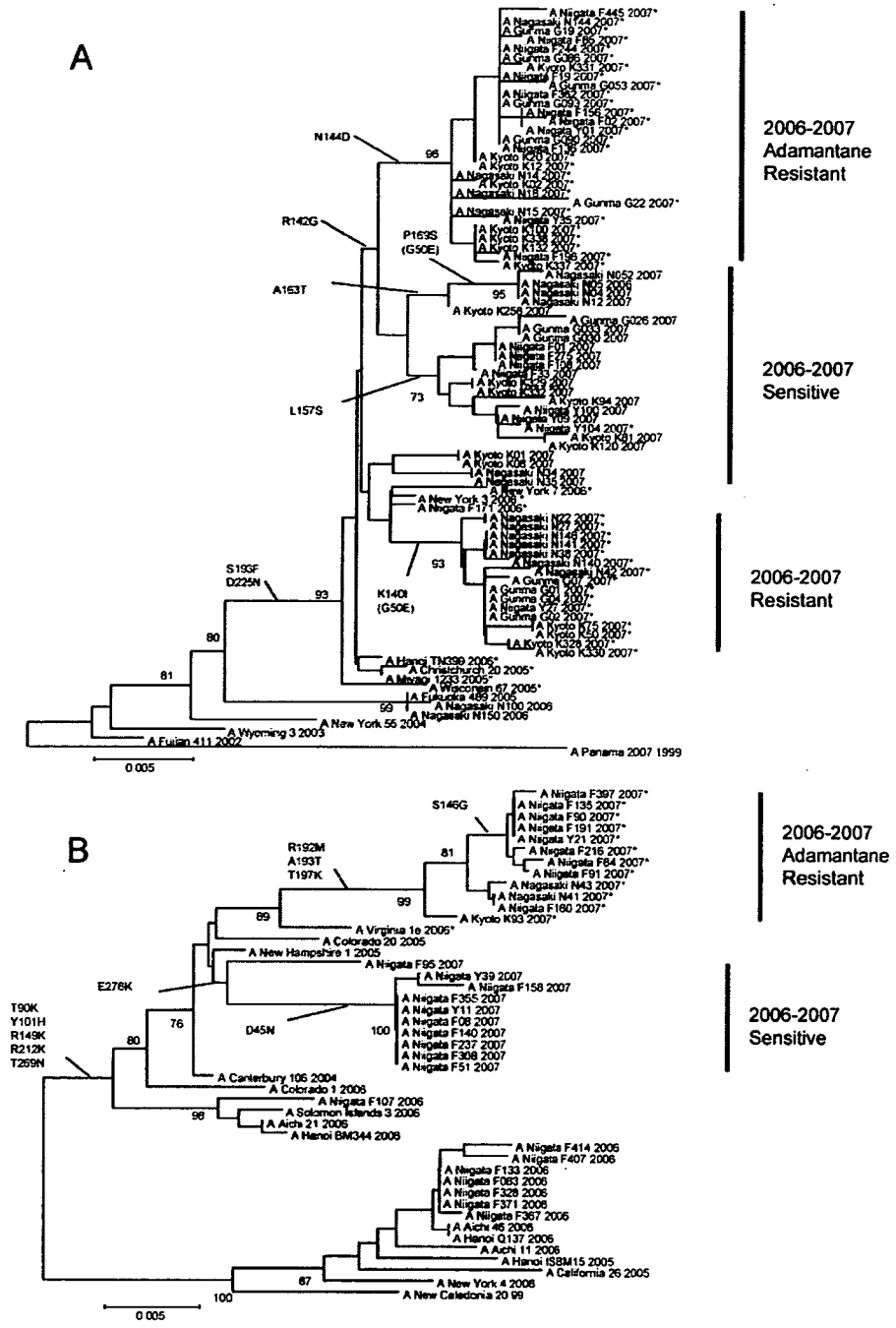

Figure. 1. Phylogenetic analysis of the hemagglutinin (HA) gene (HA1 domain) from influenza A (H3N2) and (H1N1) isolates collected in Japan during the 2006–2007 influenza season. Resistant strains are marked with asterisks; sensitive strains are unmarked. Sequences from representative vaccine components and known adamantane-sensitive or adamantane-resistant strains obtained from a genetic database (http://www.flu.lanl.gov/, Influenza Sequence Database at the Los Alamos National Laboratory; http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html, Influenza Virus resource at the National Center for Biotechnology Information) were included. The amino acid substitutions found in each group are indicated by line, and the percentage of bootstrap values over 70% is shown for major branches. *A*, A(H3N2) isolates; *B*, A (H1N1) isolates. Amino acid numbering for A (H1N1) corresponds to the H3 subtype [4, 5].

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Reply to Saito et al.

To the Editor—The rapid emergence and spread of adamantane resistance among human influenza A(H3N2) viruses circulating globally in 2004–2007 and the more recent emergence of adamantane resistance among influenza A(H1N1) viruses in certain geographical regions have been reported by several laboratories in the United States and other countries [1–6]. In their letter, Saito et al. [7] presented data on resistance to adamantanes among seasonal influenza A viruses collected in Japan during the 2006–2007 influenza season. The influenza A(H3N2) subtype was predominant in Japan during this season. Within this subtype, the incidence of adamantane resistance reached ~90%, which was a 25% increase from the previous influenza season. A drastic spike in the incidence of adamantane resistance (an increase from 0% to 64%) was also detected among influenza A(H1N1) viruses.

Our surveillance data support the view of Saito et al. [7] that the incidence of adamantane resistance among A(H3N2) viruses remains high. However, our analysis, which was conducted on a larger number of isolates from different geographic areas, revealed a significant decrease in resistance among A(H3N2) viruses isolated in many regions, compared with data from the 2005–2006 season (table 1 and previously published data [6]).

In addition to observations of Saito et al. [7] regarding influenza A (H1N1) viruses circulating in Japan, our data demonstrate that the incidence of adamantane resistance increased significantly among viruses of this subtype that were collected in 2006–2007 in other countries in Asia, as well as in Europe. In contrast, in the United States, as well as in the other countries of North and South America, the incidence of adamantane resistance among viruses of the H1N1 subtype remained nearly unchanged from the last year. These observations demonstrate a substantial geographical difference in the incidence of resistance, especially among H1N1 viruses.

Table 1. Incidence of resistance to adamantanes among inflenza A viruses collected worldwide in 2006–2007.

Region	Resistant viruses, no. (%)	
	A(H3N2)	A(H1N1)
Asia	235 (66.4)	118 (73.7)
Europe	71 (35.2)	45 (60.0)
North America	481 (68.0)	519 (2.9)
South America	77 (94.8)	29 (0)
Total	864 (67.2)	711 (18.1)

NOTE. Viruses submitted and tested at the World Health Organization Collaborating Center for Surveillance, Epidemiology, and Control of Influenza at the Centers for Disease Control and Prevention, Atlanta, Georgia.

On the basis of phylogenetic analysis of the hemagglutinin (HA) gene, we previously reported that the drug-resistant influenza A(H1N1) viruses circulating in several countries in Asia belong to a distinctive genetic group (clade 2a) [6]. In contrast, the HA sequences of the drug-sensitive A(H1N1) viruses that were collected elsewhere (in the United States, in particular) during the same period fell into genetically different groups (clades 1 and 2b). According to the report by Saito et al. [7], all drug-resistant A(H1N1) viruses collected in Japan in 2006–2007 had amino acid changes R192M, A193T, and T197K in the HA molecule. However, given our published [6] and most recent data, not all of the drug-resistant A(H1N1) viruses circulating worldwide during that season shared these 3 amino acid changes. Moreover, our previous and current data indicate that there are no signature amino acid changes in the HA

molecule that are unique to resistant A(H3N2) viruses as well.

In conclusion, we believe that several factors that are not yet well understood may contribute to the emergence and spread of adamantane-resistant viruses regionally and globally. Amantadine use in Asia during the severe acute respiratory syndrome outbreak and the avian influenza scare may have facilitated the emergence and later the spread of adamantane-resistant influenza A(H3N2) viruses in the region. However, it is unlikely to be the only factor that contributed to the alarming global spread of drug-resistant A(H3N2) viruses during 2005–2006. It is unclear whether the changes in the HA molecule alone or in combination with a novel gene constellation ('N-lineage' [8]) played a role in the unprecedented spread of drug-resistant A(H3N2) viruses in other countries where the use of amantadine and/or rimantadine was limited or nonexistent. The dramatic increase in the incidence of resistance to adamantanes among viruses of the other subtype—A(H1N1)—that are currently circulating in some Asian countries raises further concerns. It also challenges our understanding of the mechanisms that underlie viral evolution and the potential effects of drug resistance on viral fitness. Analysis of the mechanisms that drive the emergence and spread of drug resistance, as well as close monitoring of the viruses circulating in different geographic regions, are critical for confronting the global threat of epidemics or even pandemics caused by drug-resistant viruses.

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Recurrence and Persistence of Fever in Children Who Developed Amantadine-Resistant Influenza Viruses after Treatment

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In recent years, a dramatic increase of amantadine-resistant influenza A has occurred globally, but limited data have been available on the clinical course of patients developed amantadine-resistant viruses. We compared fever reduction between patients who developed resistance or remained sensitive in a pediatric clinic in Niigata, Japan, from 2000 to 2006. A total of 2,802 clinical samples were collected from patients who visited the pediatric outpatient clinic with influenza like illness during the seven influenza epidemic seasons. Patients were divided into 4 groups and analyzed for the fever reduction after amantadine treatment: emerged amantadine-resistant (n = 15); amantadine-sensitive (n = 35); patients administered no antiviral drugs (n = 42); and oseltamivir-treated patients (n = 320), which served as references. All 4 groups showed alleviation of fever up to day 3. The amantadine-resistant group had a significant recurrence of fever on day 4 and/or 5, and as a consequence, the course of illness was prolonged. Considering the pattern of fever, recurrent and persistent patterns were found significantly at higher rates in children with emerged resistant virus compared to other groups, and the age tended to be younger in amantadine-resistant compared to amantadine-sensitive group (3.9 ± 3.0 vs 6.7 ± 4.1 years old, n.s.). Therefore, we concluded that younger children were prone to develop amantadine-resistance after treatment and showed a significant recurrence of fever on day 4 and/or 5, and the course of illness was consequently prolonged. ——— influenza; amantadine; antiviral-resistance; children; recurrence of fever.
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Amantadine and rimantadine are adamantane derivatives, known as M2 channel blockers, which inhibit influenza A virus replication by blocking the M2 protein ion channel activity and thereby preventing viral uncoating and release of free ribonucleoproteins into the cytoplasm of infected cells (Pinto and Lamb 2007). Amantadine has been shown to be effective for treatment and prevention of human influenza A virus infections (Monto and Arden 1992; Oxford et al. 2003). In Japan, the drug was approved for the treatment of influenza A in November 1998.

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During the 1998-1999 through 2005-2006 influenza seasons, the prescription ranged from 0.1 to 2.7 million treatment courses, where one treatment course is equivalent to one dosage of 100 mg for 5 days per person.

Patients who were infected with influenza A virus normally shed drug-sensitive viruses during the early course of treatment. However, patients treated with amantadine or rimantadine shed drug-resistant viruses later (Hayden et al. 1989, 1991; Suzuki et al. 2003), especially after 5-7 days of therapy (Hall et al. 1987). Approximately, one-third of the patients develop such resistance (Hayden and Hay 1992; Saito et al. 2002). It has been reported that influenza A virus becomes resistant to the drug through a single amino acid substitution at positions 26, 27, 30, or 31 within the transmembrane region of the M2 gene (Pinto et al. 1992; Holsinger et al. 1994; Pinto and Lamb 2006). Viral resistance to adamantanes can confer cross resistance to both amantadine and rimantadine (Hay et al. 1986; Belshe et al. 1988).

It is generally accepted that amantadine or rimantadine resistant viruses are not more virulent or transmissible than susceptible viruses (Hayden 2006). Studies in the past documented emergence of rimantadine resistant influenza A virus after treatment (Thompson et al. 1987; Monto and Arden 1992; Saito et al. 2002), but limited data are available on the clinical significance of resistant viruses in treated patients. We therefore conducted a multiple influenza season observational study of fever reduction on patients who shed amantadine-resistant strains after therapy, in comparison with amantadine-sensitive and other treatment cases.

Materials and Methods

Study population and clinical samples

Children who visited Yoiko Pediatric Clinic in Niigata, Japan, with influenza-like illness (ILI), during seven influenza seasons from January 2000 to April 2006 were recruited. ILI was defined as a condition characterized by a sudden onset of fever (≧ 37.5°C) and respiratory symptoms, headache, arthralgia or myalgia. After obtaining written informed consent, two nasopharyngeal swabs or a certain amount of nasal aspirates were collected from patients for screening with influenza rapid tests. An aliquot, which can either be one of the two swabs taken at the same time or remaining aspirates, underwent further laboratory examinations for virus isolation and amantadine susceptibility testing. Influenza rapid test kits used in the study were such as QuickVue Rapid SP influ (DS Pharma Biomedical Co., Ltd., Osaka), Espline Influenza A&B-N (Fujirebio Inc., Tokyo) and Quick S-Influ A/B "SEIKEN" (Denkaseiken Co., Ltd., Tokyo).

Amantadine was administered to patients diagnosed as positive for the influenza A virus by rapid antigen testing. The drug was given within 48 hrs of onset at a dosage of 5 mg/kg body weight/day (maximum dosage of 100 mg/day). Influenza A patients who did not undergo amantadine therapy were given oseltamivir as a reference twice daily at a dosage of 150 mg per day for patients weighing ≧ 37.5 kg, or 4 mg/kg/day for patients weighing < 37.5 kg. The decision on whether to administer amantadine or oseltamivir was left to the discretion of the pediatrician, who considered the background and characteristics of the patients such as the presence of other existing diseases, patient age, and patient preference. Patients' information such as age, sex, body temperature on the first visit, time of onset, history of influenza vaccination, name of antiviral drug administered and treatment period were recorded by the pediatrician. Each patient was given a diary card to record axillary temperature three times daily (9 a.m., 12 noon, and 8 p.m.) at home for up to eight days, and these diary cards were returned by mail or brought to the clinic. Amantadine-treated patients were requested to visit the clinic 3-5 days later and to allow collection of second clinical samples. This study was approved by the Medical Faculty Ethics Committee of the Niigata University Graduate School of Medical and Dental Sciences.

Virus isolation and amantadine susceptibility test

Nasopharyngeal swabs or aspirates from patients were suspended in viral transport media and kept at 4°C, then transferred within 7 days to the Division of Public Health, Graduate School of Medical and Dental Sciences, Niigata University, Niigata City, Japan. Supernatants of nasopharyngeal swabs or aspirates were inoculated into Madin-Darby canine kidney (MDCK) cells for influenza virus isolation. Types of viruses were determined by hemagglutination inhibition tests with influenza vaccine strain antisera for the respective seasons (Masuda et al.

2000). Amantadine susceptibility tests were performed with two series of 10-fold dilutions of viruses from cytopathic effect (CPE)-positive cultures, plated in triplicate in 96-well microplates on MDCK cells, with one dilution series containing 2.0 μg/ml of amantadine in the medium (Masuda et al. 2000). Amantadine-resistant strains were identified when less than 1.0-fold difference in log $TCID_{50}$/0.2 ml titer was observed between series of rows with and without the drug after 48 hrs of incubation at 37.0°C.

PCR (polymerase chain reaction) detection and sequencing of the M2 gene

After viral RNA was extracted from patients' nasopharyngeal swabs or isolates, reverse transcription was performed using random primers to create complementary DNA. Nested PCR was performed using specific primers to amplify the M2 region of influenza A (Masuda et al. 2000). The PCR products were sequenced to examine mutations at positions 26, 27, 30, or 31 in the transmembrane region of the M2 gene that are known to confer resistance. Finally, amantadine resistance was diagnosed from the M2 gene sequencing results.

Analysis of fever reduction

Patients enrolled in this study were divided into three groups by therapy: patients who received amantadine, those who received non-antivirals, and those who received oseltamivir. Furthermore, patients who received amantadine were subdivided into amantadine sensitive and emerged amantadine resistance after therapy. Maximum axillary temperatures on each day were evaluated in the four groups, and reduction of fever was analyzed.

Each clinical course was classified into three patterns: "good response pattern" which was defined as alleviation of the fever by day 5 with a body temperature of less than 37.8°C after starting the therapy; "recurrent pattern" which was rebound fever with a temperature greater than or equal to 37.8°C after reduction of temperature below 37.8°C until day 5; "persistent pattern" was defined by persistence of fever with a body temperature greater than or equal to 37.8°C for more than 5 days. Proportion of recurrent and persistent patterns were calculated and compared in the four study groups.

Statistical analysis

To compare mean values between the two groups, the Student's *t*-test was performed. In case of more than 2 groups, firstly analysis of variance (ANOVA) was employed, then, if statistical significance was determined by ANOVA, the Scheffe's test was performed as an *ad hoc* test. To compare median, the Kruskal Wallis method was performed. To compare proportions, chi-square test was used. p values less than 0.05 were employed to define statistical significance.

Results

Amantadine-sensitive influenza A cases

A total of 2,802 patients who visited the pediatric outpatient clinic with ILI during the seven influenza epidemic seasons were screened. Patients who did not meet the study criteria were excluded from the analysis for reasons as shown in Fig. 1. Among 50 amantadine recipients, resistant strains after treatment were detected from 15 (30.0%) recipients ("resistant group") and sensitive strains from 35 (70.0%) ("sensitive group"). Furthermore, 320 oseltamivir recipients ("oseltamivir-recipient group") and 42 non-antiviral recipients ("non-antiviral group") were included in the analysis.

Demographic details of the study groups

Sex distribution, average age, and body temperature at the first clinic visit did not differ significantly among the study groups (Table 1). Average time to clinic visit in the non-antiviral group was significantly longer than that in the oseltamivir recipient group. Vaccination status varied among the four groups. No significant differences were found in the amantadine treatment period between the sensitive and resistant groups (3.4 ± 0.7 days and 3.4 ± 0.5 days, respectively) and the time from the first to second sampling (3.4 ± 1.3 days and 3.7 ± 0.8 days, respectively).

Analysis of fever reduction in emerged amantadine-resistant cases after treatment

We examined the effectiveness of therapy among the 4 groups (Fig. 2). No significant variation in body temperature was found on day 1. On day 2 and 3, reduction of fever was observed in each group, and the maximum body temperature in the resistant group was higher than that in the sensitive group, although statistical significance

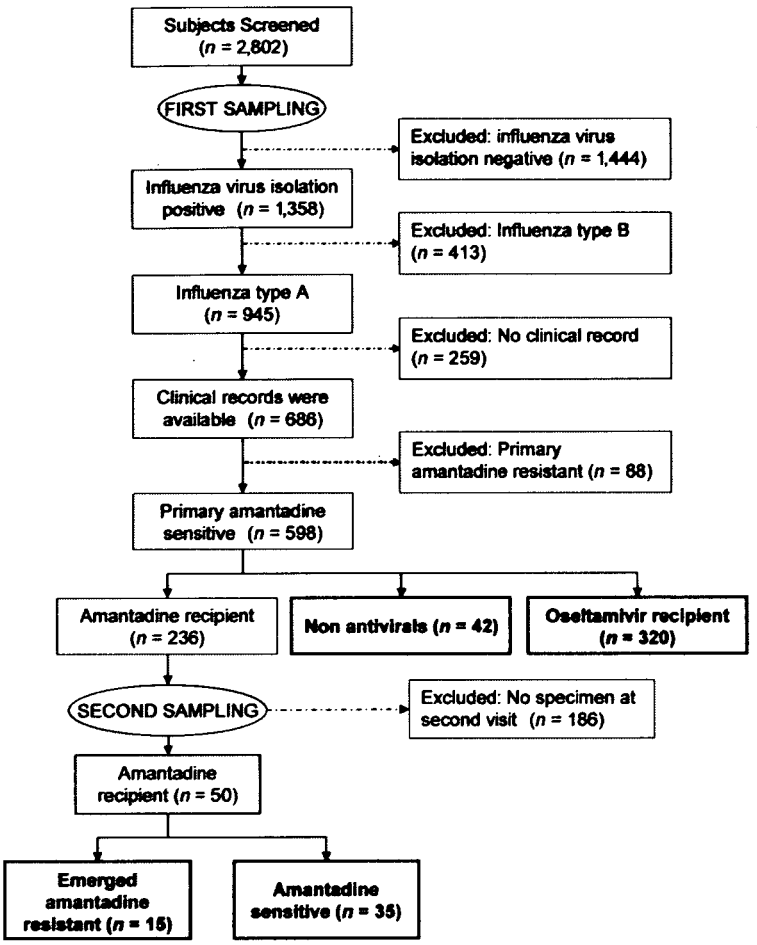

Fig. 1. Flow chart of patients employed in the study. Four study groups were indicated in boldface.

was not shown. The maximum body temperatures in the non-antiviral group (38.2 ± 1.1°C on day 2, and 37.4 ± 1.0°C on day 3) were significantly higher than in the oseltamivir-recipient group (vs 37.7 ± 0.9°C on day 2, $p < 0.05$, and vs 36.9 ± 0.7°C on day 3, $p < 0.01$).

A significant elevation of body temperature was seen in the resistant group on day 4. Average body temperature on day 4 was significantly higher in the resistant group (37.9 ± 0.9°C) than in the sensitive group (vs 37.1 ± 0.9°C, $p < 0.01$), the oseltamivir-recipient group (vs 36.7 ± 0.6°C, $p < 0.01$) and the non-antiviral group (vs 37.1 ± 0.9°C, $p < 0.01$). Fever on day 5 in the resistant group (37.7 ± 1.1°C) was also higher than in the sensitive group (vs 37.2 ± 0.9°C, $p = 0.16$) and the oseltamivir-recipient group (vs 36.7 ± 0.7°C, $p < 0.01$). However, on day 6, body temperature in the resistant group had resolved (37.1 ± 1.0°C) and no difference was found compared to other groups.

We classified all fever records from the patients into three patterns in terms of fever reduction: good response, recurrence, and persistence. Many of the children in the resistant group were classified as recurrent (40.0%) or persistent (26.7%) pattern groups (Fig. 3). In the sensitive, non-antiviral and oseltamivir recipient groups, a recurrent pattern accounted for 22.9%, 16.7%, and 3.1% respectively; and persistent pattern was

TABLE 1. Demographic characteristics of patients' groups

Characteristic	Study groups				
	Emerged amantadine resistant [1] (n = 15)	Amantadine sensitive [1] (n = 35)	Non antivirals (n = 42)	Oseltamivir recipient (n = 320)	p value
Sex, no. of male (%)	11 (73.3)	19 (54.3)	21 (50.0)	182 (56.8)	0.47 [2]
Age, mean ± S.D. (years)	3.9 ± 3.0	6.7 ± 4.1	5.5 ± 5.1	5.8 ± 4.2	0.19 [3]
Body temperature at first clinic visit, mean ± S.D. (°C)	38.8 ± 0.8	38.6 ± 0.9	38.6 ± 0.9	38.7 ± 0.8	0.91 [3]
Time from onset to clinic visit, median (range) (hrs)	11.0 (3 - 42)	22.0 (3 - 64)	24.0 (0 - 90)	17.0 (0.5 - 66)	< 0.05 [4]*
Vaccination, no. of patients who received influenza Vaccination in the season (%)	1 (6.7)	0 (0.0)	7 (16.7)	136 (42.5)	< 0.001 [2]
Amantadine treatment period, mean ± S.D. (day)	3.4 ± 0.5	3.4 ± 0.7	-	-	1.00 [5]
Oseltamivir treatment period, mean ± S.D. (day)	-	-	-	3.7 ± 0.6	-
Time from first to second sampling, mean ± S.D. (day)	3.7 ± 0.8	3.4 ± 1.3	-	-	0.33 [5]

[1] Amantadine susceptibility, confirmed with specimens at the second sampling.
[2] Chi-square test was used for comparison among the groups.
[3] Analysis of variance was used for comparison among mean values of each group.
[4] Kruskal Wallis method was used for comparison among median values of each group.
[5] Student's t-test was used for comparison between two groups.
*Statistically significant difference in median values between the non-antiviral group and the oseltamivir recipient group.

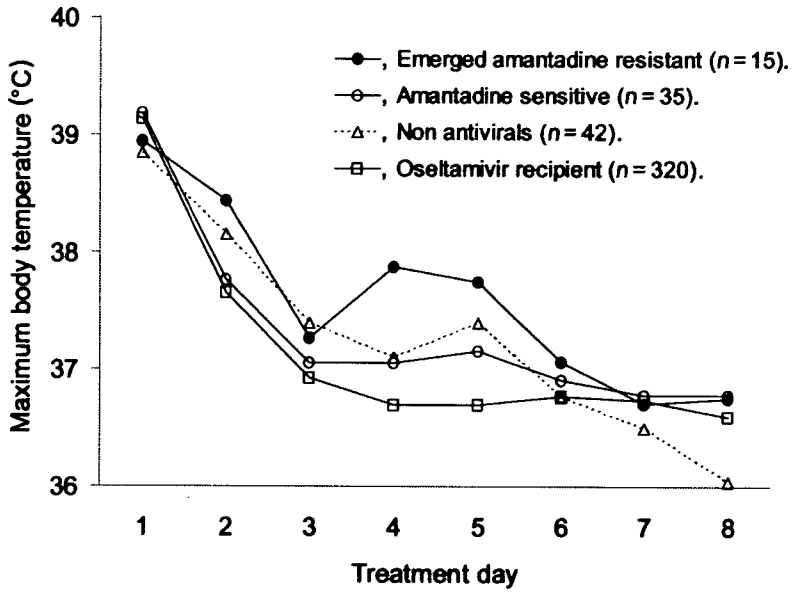

Fig. 2. Daily average maximum body temperatures in the study groups.
The four study groups were as follows: amantadine recipients shedding resistant viruses (●, n = 15); amantadine recipients shedding sensitive viruses (○, n = 35); patients who received no antivirals (Δ, n = 42); and oseltamivir recipients (□, n = 320). On day 4, the emerged amantadine-resistant vs the other three groups, $p < 0.01$, respectively. On day 5, the emerged resistant group vs the oseltamivir recipients, $p < 0.01$. On day 2 and 3, the non-antiviral group vs the oseltamivir recipients, $p < 0.05$ and $p < 0.01$, respectively.

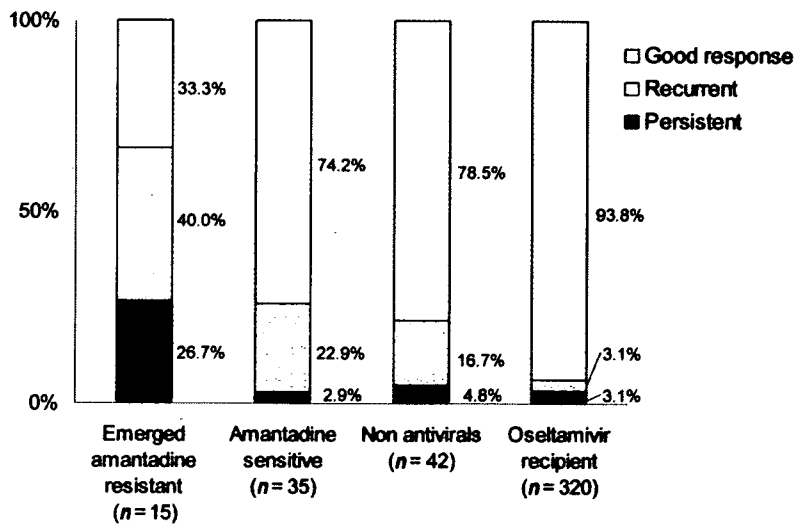

Fig. 3. Proportion of the three patterns of fever reduction among the four groups.
"Good response" is rapid fever reduction group; "recurrence" fever reduction at first but recurrent fever later; and "persistence" group.

2.9%, 4.8%, and 3.1% respectively. Combined proportion of persistent and recurrent patterns in the amantadine-resistant group was significantly higher (66.7% [10 of 15]) than in the sensitive group (vs 25.7% [9 of 35], $p < 0.01$), the non-antiviral group (vs 21.4% [9 of 42], $p < 0.01$), and the oseltamivir-recipient group (vs 6.3% [20 of 320], $p < 0.01$).

Individual fever records showed that in the resistant group, 5 patients (average age 6.4 years

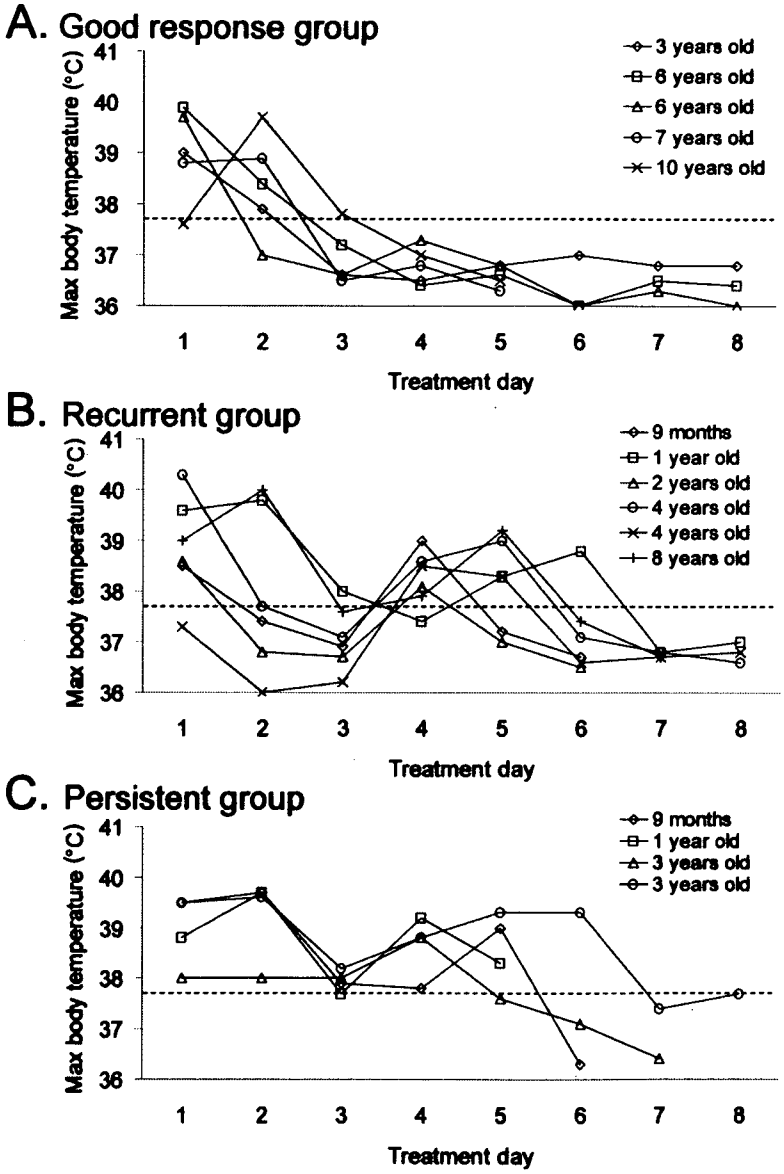

Fig. 4. Individual records of daily maximum body temperature in children who shed amantadine-resistant viruses after treatment.
Good response (panel A) was defined as rapid fever reduction < 37.8°C by day 5. Recurrent group was defined as fever reduction < 37.8°C in the early course of the illness, but a recurrence ≧ 37.8°C by day 5 (panel B). Persistent group was defined as showing persistence of fever ≧ 37.8°C by day 5 without alleviation (panel C). Horizontal dotted lines indicate 37.8°C.

old) showed good response patterns (Fig. 4A), while 6 patients (average age 3.3 years old) exhibited recurrent patterns (Fig. 4B), and 4 children (average age 1.9 years old) showed persistent patterns (Fig. 4C).

Among 50 amantadine treated children (35 amantadine-sensitive and 15 emerged amantadine-resistant), 41 were A/H3N2 subtype and 9 were A/H1N1. Thirteen (31.7%) of 41 A/H3N2 and 2 (22.2%) of 9 A/H1N1 were resistant, but the frequency of resistance between A/H3N2 and A/H1N1 was not statistically significant.

As to timing of recovery, resistant influenza viruses were collected on day 3 from 8 children (53.3%), 4 patients (26.7%) on day 4, and 3 patients (20.0%) on day 5 after starting the amantadine therapy. In the M2 gene sequence analyses, seven (46.7%) out of 15 amantadine-resistant virus had a change at position 31 (serine to asparagine), five (33.3%) at 27 (valine to alanine), two (13.3%) at 30 (alanine to threonine). One strain (6.7%) had dual mutations at 31 (serine to asparagine) and at 27 (valine to alanine). However, no significant difference was observed in clinical pictures by the positions of mutation.

Discussion

In this observational study, the course of influenza illness differed between patients shedding amantadine-resistant and sensitive strains. All four study groups showed reduction of fever during the first few days. The amantadine-resistant group showed a significant recurrence of fever on day 4 and/or 5, and as a consequence, the course of illness was prolonged. In an earlier study (Hall et al. 1987), illness severity which scored late in therapy tended to be higher in rimantadine-treated children who shed resistant viruses compared to those who did not, but statistical significance was not demonstrated. Furthermore, another study (Hayden et al. 1991) indicated that average temperatures did not differ between the rimantadine groups over the first 4 days of treatment, but a non-significant elevation of temperature appeared that shed resistant virus on treatment day 5. Thus, the present study is the first to show significant recurrence and persistence of fever in children who shed resistant influenza viruses after treatment.

Considering the pattern of fever reduction, recurrent and persistent patterns were found significantly at higher rates in children in the resistant group compared to other groups, and the age tended to be younger in the resistant group compared to the sensitive group. These findings are considered as the clinical feature of children who developed amantadine-resistant influenza A viruses. Furthermore, in the emerged resistant group, age of children who showed persistent and recurrent patterns were younger than good response pattern group. In a study of oseltamivir, resistant viruses to this drug appeared more often in young children (Kiso et al. 2004), and it was explained that younger children experiencing their first or second influenza infections typically manifest a prolonged period of illness and virus shedding, and possess higher virus titer. In general, younger children do not have immunological memories in their T and B cells because of no prior exposure of any types of influenza (Ahmed and Rouse 2006; Kalia et al. 2006), and thus, their immune response is slower than that of adults and elderly. Consequently, high viral load in children may allow greater opportunity for selection of resistant viruses after treatment of amantadine.

To reduce the emergence of resistant strains, amantadine therapy is advised to be discontinued as soon as clinically warranted, generally after 3-5 days of treatment, or within 24 hrs after the disappearance of signs and symptoms. The dosage recommended in the United States is 5 mg/kg/day, and should not exceed 150 mg in two divided dosages for children aged 1-9 years. In this study, due to the Japanese regulations, the daily dosage was lower than that in the United States and the duration of treatment was shorter, which was 3 to 4 days. While recurrence of fever was observed on day 4, and most of the emerged amantadine-resistant viruses (84.3%) were recovered until day 4, we may not rule out the supposition that this fever aggravation was caused by the shorter period of treatment. However, this assumption can not explain the fever difference between the resistant and sensitive groups, since both groups pos-

sessed similar treatment durations. Thus, we assume that the fever difference between the two groups is linked with amantadine susceptibility status. Further studies are needed to determine whether recurrence and persistence of fever in individuals shedding drug resistant strains are associated with increased viral load due to development of resistance. In this study, $TCID_{50}$ of the amantadine resistance and sensitive groups at the second sampling were 3.7 and 5.3 (data not shown), respectively. However, these results did not reflect the true viral titers in the original samples, because the virus titer was measured after three passages in MDCK cells. Therefore, further specific study such as quantitative real-time PCR is warranted.

In order to determine the clinical significance of drug-resistant virus from treated patients, reduction of fever and improvement of daily scores for symptoms and severity of illness was used in the previous studies (Hall et al. 1987; Thompson et al. 1987; Hayden et al. 1989, 1991). For this paper, only temperature data but not other symptoms was analyzed since it was the only objective measurement that was not affected by biases from doctors' or participants' feelings or judgments. We used the maximum body temperature data of patients with 3 measurements per day to calculate average maximum body temperatures in each group, so as to avoid possible influence of temporary antipyretic use, which was administered for patients when the fever was too high (*e.g.* > 38°C).

In Japan, more than 90% of influenza cases in children are administered antiviral drugs, mostly oseltamivir and occasionally amantadine (Sugaya et al. 2007). In this study, only 42 of 424 influenza patients (9.9%) did not receive antiviral drug (non-antiviral group). The reasons for no receipt were negative result with rapid test in ambulatory, guardian's will, or more than 48 hrs had passed from onset to clinic visit. Thus, the average time from onset to clinic visit in the non-antiviral group was the longest among the four groups.

In this study, a subset of amantadine recipients (186 of 236) were not included due to lack of a second sample. This might suggest that these patients did not return to the clinic because of adequate recovery from their illness. However, reviewing their clinical records, a combined proportion of the recurrent and persistent pattern rates did not reveal any significant difference between with or without second samples (data not shown). Thus, patients did not return for a second sampling due to unknown reasons not related to their recovery.

Proportion of recovering resistant strains was higher in those shedding A/H3N2 strain than in those shedding A/H1N1 strain although statistically not significant, and these results supported our previous report on a difference of resistant strain appearance by subtype (Saito et al. 2003). Furthermore, we could not find any relationship between clinical pictures and positions of mutation in the M2 gene.

In this study, 88 primary amantadine-resistant cases were excluded, and most of them were A/H3N2 viruses with the S31N mutation in the 2005/06 season reported as a clade N, which was related to a dramatic increase of resistance in communities in Japan (Saito et al. 2006; Saito and Suzuki 2007), Asia, and North America (Barr et al. 2007; Deyde et al. 2007). Further investigations on clinical courses with primary amantadine-resistant viruses are warranted, since available information on concordance or discordance between clinical data and phenotypic/genotypic assays in antiviral resistance is limited.

In conclusion, younger children tended to develop amantadine-resistance after treatment, and these children showed higher incidence of persistence or recurrence of fever on day 4 and/or 5.

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Effectiveness of Oseltamivir Treatment among Children with Influenza A or B Virus Infections during Four Successive Winters in Niigata City, Japan

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Oseltamivir has been used for treatment of influenza A and B infections, but recent reports documented that it was less active against the latter. We compared the effectiveness of oseltamivir in children between laboratory confirmed influenza A and B over 4 influenza seasons from 2001 to 2005 in a pediatric clinic in Japan. Among 1,848 patients screened, 299 influenza A and 209 influenza B patients were administered oseltamivir (treated groups), and 28 influenza A and 66 influenza B patients were assigned as non-treated groups. The duration of fever, defined as period when patients had the maximum temperature higher than 37.5°C in three-time measurements in a day after the clinic visit, was evaluated among the four groups. In uni-variate analysis, the duration of fever was shorter for treated group than non-treated for influenza A (1.8 ± 0.9 days vs 2.6 ± 1.3 days, $p < 0.01$), but it was not significant for influenza B (2.4 ± 1.3 days vs 2.8 ± 1.2 days, $p = 0.9$). The fever duration was longer in treated influenza B than A patients ($p < 0.01$). Multi-variate analysis indicated younger age (< 6 years old) and higher body temperature at the clinic visit prolonged the duration of fever. Adjusted average duration of fever indicated that oseltamivir was effective for both types, but more effective on influenza A, and the benefit increased for younger children. Our data provide evidence that oseltamivir is beneficial for influenza infections, but the effectiveness is differed by type and age.

——— influenza; anti-viral drugs; oseltamivir; children; effectiveness.
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Influenza outbreaks occur annually across the world, causing excess morbidity and mortality (Simonsen et al. 2000; Nicholson et al. 2003; Centers for Disease Control and Prevention 2006). For influenza treatment, there are two types of anti-influenza drug: amantadine and neuraminidase inhibitors (oseltamivir and zanamivir) (Monto 2003; Oxford et al. 2003; Moscona 2005; Oxford 2005; Jefferson et al. 2006). Amantadine is effective for treatment of influenza

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A virus infections while neuraminidase inhibitors are for both influenza A and B (Treanor et al. 2000). Early treatment reduces the severity and duration of influenza illness and associated complications (Nicholson et al. 2000; Aoki et al. 2003; Kawai et al. 2005).

The neuraminidase inhibitors, zanamivir and oseltamivir, interfere with the release of progeny influenza viruses from infected host cells and spread to neighboring cells in the respiratory tract. Clinical efficacy of oseltamivir has been established as treatment for influenza in adults (Hayden et al. 1999; Nicholson et al. 2000; Treanor et al. 2000) and children (Whitley et al. 2001). The neuraminidase inhibitors were tested to be less active against influenza B than A viruses in vitro studies (Boivin and Goyette 2002; Aoki et al. 2003; Mungall et al. 2004). Moreover, increasing evidence suggests that oseltamivir is less effective against influenza B than influenza A infections (Kawai et al. 2006; Sugaya et al. 2007). The present study was conducted to evaluate the efficacy of oseltamivir treatment among children with influenza A and B virus infections during four successive winters in Japan using uni-variate and multi-variate analysis adjusted for various factors that affect the course of illness.

Materials and Methods

Study population and laboratory methods

This study was conducted during 4 influenza seasons from November 2001 to May 2005 at a private pediatric outpatient clinic located at the city center in Niigata City, Japan with a total population of approximately 500,000. This clinic had no bed facility, and approximately 2,300 outpatients visited per month.

Influenza-like illness was defined on the basis of a sudden fever (≧ 37.5°C) and any acute respiratory symptoms and signs, such as, cough, rhinorrhea, sneezing, wheezing, sore throat, headache, nausea, or malaise. Nasopharyngeal swabs or aspirates were examined with rapid antigen test kits for diagnosis of influenza A or B prior to antiviral drug treatment (oseltamivir or amantadine) at the initial office visits. Influenza rapid test kits, such as QuickVue Rapid SP influ (DS Pharma Biomedical Co., Ltd., Osaka), Espline Influenza A&B-N (Fujirebio Inc., Tokyo), and Quick S-Influ A/B "SEIKEN" (Denkaseiken Co., Ltd., Tokyo) were used to screen influenza A or B infections.

Patients were assigned to influenza treatment or non treatment groups, depending whether or not they want to receive antiviral drug medication according to the rapid test results. For patients with influenza A, the decision of whether to receive antivirals (oseltamivir or amantadine) or not was left to patients or their family. For influenza B, patients could choose either oseltamivir or no antiviral therapy. The two drugs were administered twice daily (oseltamivir, 150 mg per day for children ≧ 37.5 kg in weight; or 4 mg/kg for children with < 37.5 kg: amantadine, 1.5-2.5 mg/kg). Both drugs were prescribed for 5 days. For some patients, administration of drugs was discontinued if symptoms were alleviated within 5 days. Single use of antipyretics was allowed when a child had a fever more than 38.5°C.

Written informed consent was acquired from parents of patients to obtain clinical information and specimens for virological investigations upon enrollment to the study. Age, sex, body weight, vaccination status, use of antipyretics, type of drug, the time from the onset of fever to the administration of anti-influenza drug, body temperatures, and the results of rapid antigen test kits were recorded for all patients by the clinician at the time of report to the clinic. The parents were given a diary card to record body temperatures 3 times daily (at 9:00, 12:00 and 20:00 o'clock) and any symptoms such as cough, rhinorrhea, sore throat, fatigue, appetite loss, myalgia, vomiting, or diarrhea, occurring up to 5 days after the therapy started. Parents were requested to return the card by visiting or mailing to the clinic after completion of the course. Time until treatment was defined as days from fever onset until the clinic visit.

Nasopharyngeal swabs or aspirates were collected from the patients, placed in viral transport medium, and then transferred to the Department of Public Health, Niigata University Graduate School of Medical and Dental Sciences. The samples were stored at 4°C for a few days until viral culture, and aliquots were kept at −80°C. For virus isolation, supernatants of specimens were inoculated into Madin-Darby canine kidney cells. Types and subtypes were determined by hemagglutination inhibition tests with type-specific antisera (Masuda et al. 2000). Detection of the influenza genome was performed by reverse transcription-polymerase chain reaction (RT-PCR) (Saito et al. 2002). Briefly, viral RNA was extracted from nasopharyngeal aspirate specimens and reverse transcription reactions were performed for complementary DNA synthesis as described previously