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

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

Methods

Patients

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

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

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

Antigen detection test kits and virus isolation

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

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

NA inhibition assay

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

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

NA sequence analysis

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

Statistical analysis

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

Results

Patient characteristics and H275Y mutation before therapy

Of 158 patients with influenza A(H1N1) virus infection, 44 presented during the 2007–2008 season (December 1, 2007–February 27, 2008), 32 during the 2008–2009 season (December 1, 2008–April 30, 2009), and 82 during the 2009–2010 season (November 1, 2009–April 30, 2010). No H275Y mutation was detected before therapy by NA sequence analysis in seasonal H1N1 in 2007–2008 or in H1N1pdm in 2009–2010, but in all seasonal H1N1 in 2008–2009. Patient demographic characteristics for seasonal H1N1 without H275Y mutation (2007–2008), seasonal H1N1 with H275Y mutation (2008–2009), and H1N1pdm (2009–2010) are summarized in Table 1. No significant pretreatment differences among the groups were found for median values of age, body temperature, or total symptom score, male-to-female ratio, or vaccination status. The median (25th–75th percentile) time from onset of symptoms to sampling was 13.8 (7.1–22.1) h in the 2007–2008, 19.7 (12.8–29.9) h in the 2008–2009, and 19.8 (14.0–26.9) h in the 2009–2010 seasons (2007–2008 vs. 2008–2009, $P = 0.071$; 2007–2008 vs. 2009–2010, $P = 0.010$; 2008–2009 vs. 2009–2010, $P = 0.962$).

Body temperature before and after the start of therapy

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

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

Persistence of other symptoms after therapy

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

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

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

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

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

BT body temperature

[a] Median (25–75 percentile)

[b] Vaccination, vaccination for seasonal influenza

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

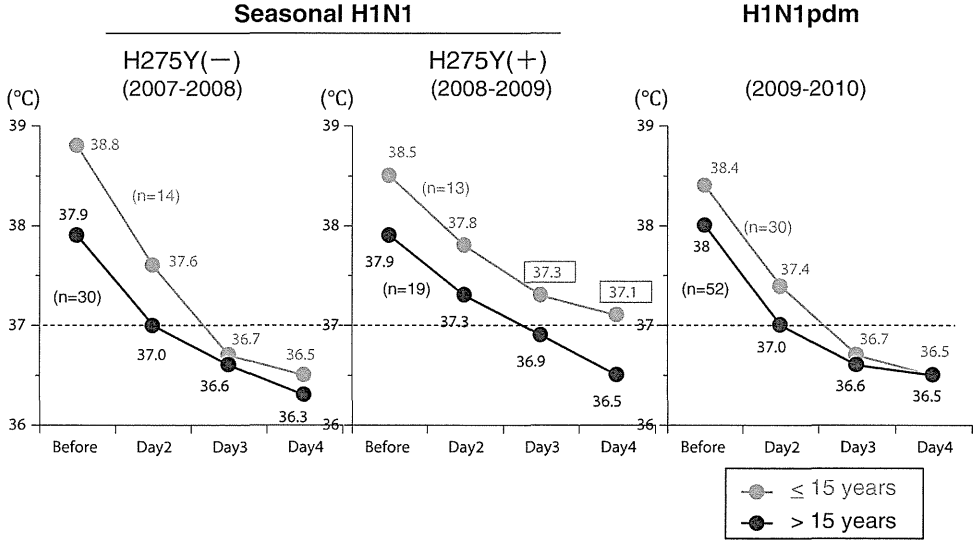

Fig. 1 Mean body temperature before, and on days 2, 3, and 4 after, the start of therapy in the 2007–2008, 2008–2009, and 2009–2010 seasons. Mean body temperature above 37.0°C was seen not only before day 2, but also on day 3 or 4 (*numbers enclosed in boxes*) in children in the 2008–2009 season in which H1N1 with the H275Y mutation prevailed

The persistence rates for cough were 7.1% (1/14), 46.2% (6/13), and 16.7% (5/30), respectively ($P = 0.033$ between 2007–2008 and 2008–2009), in children. The rates for adults were 20.0% (6/30), 42.1% (8/19), and 23.1% (12/52), respectively (NS among the three groups).

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

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

Virus persistence after oseltamivir therapy

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

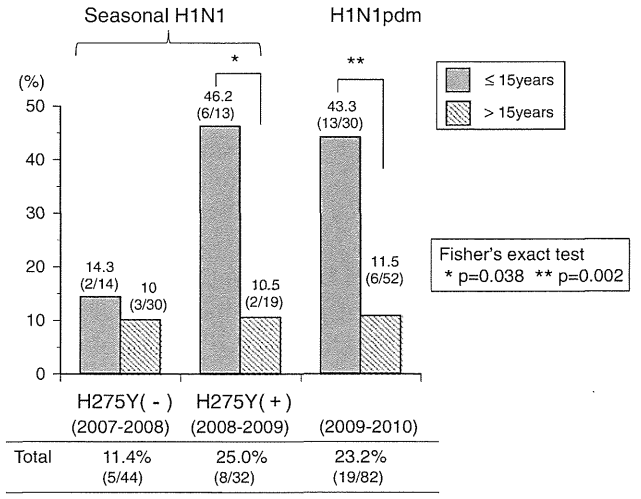

Fig. 2 Persistence rate of virus on the 4th–6th days after the start of therapy. The rate was significantly higher for children ≤15 years (*solid bars*) than for adults 15 years and older (*hatched bars*) in both 2008–2009 and 2009–2010 seasons

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

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

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

H275Y mutation after therapy and IC_{50}

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

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

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

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

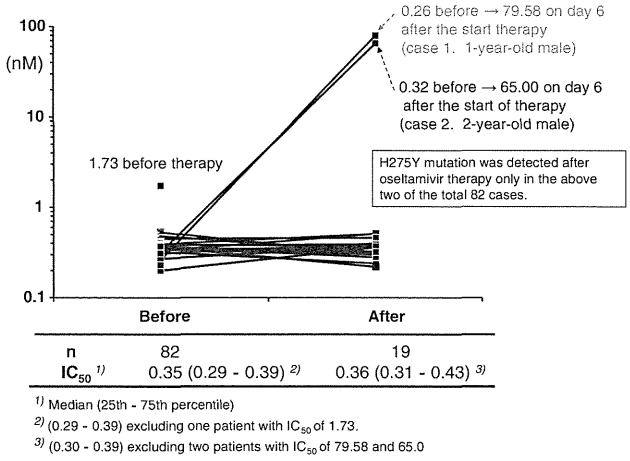

Fig. 3 IC_{50} for oseltamivir before and on days 4–6 after the start of therapy for patients with pandemic H1N1 in the 2009–2010 season. IC_{50} for oseltamivir was increased approximately 200- to 300 fold in two patients in whom the H275Y mutation emerged

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

Discussion

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

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

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

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

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

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Identification of Oseltamivir Resistance among Pandemic and Seasonal Influenza A (H1N1) Viruses by an His275Tyr Genotyping Assay Using the Cycling Probe Method▽

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Neuraminidase inhibitors are agents used against influenza viruses; however, the emergence of drug-resistant strains is a major concern. Recently, the prevalence of oseltamivir-resistant seasonal influenza A (H1N1) virus increased globally and the emergence of oseltamivir-resistant pandemic influenza A (H1N1) 2009 viruses was reported. In this study, we developed a cycling probe real-time PCR method for the detection of oseltamivir-resistant seasonal influenza A (H1N1) and pandemic influenza A (H1N1) 2009 viruses. We designed two sets of primers and probes that were labeled with 6-carboxyfluorescein or 6-carboxy-X-rhodamine to identify single nucleotide polymorphisms (SNPs) that correspond to a histidine and a tyrosine at position 275 in the neuraminidase protein, respectively. These SNPs confer susceptibility and resistance to oseltamivir, respectively. In the 2007-2008 season, the prevalence of oseltamivir-resistant H1N1 viruses was 0% (0/72), but in the 2008-2009 season, it increased to 100% (282/282). In the 2009-2010 season, all of the pandemic influenza A (H1N1) 2009 viruses were susceptible to oseltamivir (0/73, 0%). This method is sensitive and specific for the screening of oseltamivir-resistant influenza A (H1N1) viruses. This method is applicable to routine laboratory-based monitoring of drug resistance and patient management during antiviral therapy.

The neuraminidase (NA) inhibitors (NAIs) oseltamivir and zanamivir are currently the antiviral drugs of choice for treatment and prophylaxis of influenza virus infections. NAIs prevent the release and spread of progeny virions from infected cells (16). A major concern is the emergence of drug-resistant strains during antiviral therapy. Oseltamivir-resistant viruses possessed a histidine-to-tyrosine amino acid substitution at position 275 in type N1 NA protein (His274Tyr in N2 numbering). This mutation was initially detected in patients who were infected with seasonal influenza A (H1N1) viruses after oseltamivir treatment (10). The prevalence of oseltamivir resistance was low in the 2007-2008 season, but a sudden increase was reported in the following season, when the His275Tyr mutants spread globally and were the predominant strain among seasonal H1N1 viruses (23).

In the spring of 2009, pandemic influenza A (H1N1) 2009 virus (H1N1pdm) emerged and circulated worldwide (4). Initial reports showed that all H1N1pdm viruses were sensitive to neuraminidase inhibitors, and recently, so far only 298 cases of oseltamivir-resistant H1N1pdm viruses possessing the His275Tyr mutation were reported by the Centers for Disease Control and Prevention and the World Health Organization (2, 3, 24). The majority of His275Tyr mutations in H1N1pdm viruses were detected after therapeutic or preventive administration of oseltamivir. Although the proportion of oseltamivir-resistant H1N1pdm viruses is low at the moment, continued monitoring for oseltamivir-resistant viruses is important because of the possibility that the prevalence of these resistant strains may increase, which happened among the contemporary seasonal H1N1 viruses (1, 20, 23).

Various high-throughput methods used in detecting the His275Tyr mutation among oseltamivir-resistant H1N1pdm viruses include pyrosequencing (7, 25), real-time PCR method using a TaqMan probe, and the rolling circle amplification (RCA) technology (12, 21, 22). Cycling probe real-time PCR is an alternative method that employs a sequence-specific chimeric probe in detecting single nucleotide polymorphisms (SNPs) (19). We previously applied this method to identify amantadine-resistant seasonal influenza A (H1N1) and A (H3N2) viruses with the Ser31Asn mutation in the M2 channel protein (19). We showed rapid detection of the Ser31Asn mutation from nasopharyngeal swabs in several hours by this method and demonstrated its high sensitivity and specificity, which are comparable to those of the gene sequencing method. In the study described in this report, we designed new sets of primers and probes to identify the His275Tyr mutation in NA which confers oseltamivir resistance, and we investigated the

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TABLE 1. Primers and probes for cycling probe real-time PCR method

Subtype	Primer or probe	Sequence (5′–3′)	Location[a]
Seasonal influenza A (H1N1) virus	sH1N1-His275Tyr forward primer	5′-CAAGATCGAAAAGGGGAAG-3′	768–786
	sH1N1-His275Tyr reverse primer	5′-GACACCCAAGGTCGATTTG-3′	896–914
	sH1N1-His275[b]	5′-(Eclipse[c])-[AT*G*][d]AAAATTGGGTG-(FAM[e])-3′	812–825
	sH1N1-Tyr275[b]	5′-(Eclipse)-[AT*A*]AAAATTGGGTG-(ROX[e])-3′	812–825
Influenza A pandemic (H1N1) 2009	H1N1pdm-His275Tyr forward primer	5′-TGGACAGGCCTCATACAAGA-3′	744–763
	H1N1pdm-His275Tyr reverse primer	5′-GCCAGTTATCCCTGCACACA-3′	870–889
	H1N1pdm-His275[b]	5′-(Eclipse)-CCTAATTAT[C*A*C]T-(FAM)-3′	814–826
	H1N1pdm-Tyr275[b]	5′-(Eclipse)-AT[T*A*C]TATGAGGA-(ROX)-3′	821–833

[a] Location of primers and probes in the NA-coding region (total, 1,413 bp), segment 6, of influenza A (H1N1) virus. Note that both cycling probes for seasonal H1N1 were designed as reverse complements.
[b] Fluorescent dye and quencher-labeled DNA/RNA chimeric probe.
[c] Quenching molecule.
[d] Nucleotides inside brackets indicate the codon relevant to sequences for oseltamivir sensitivity (His) and resistance (Tyr). Boldface and italicized letters indicate the nucleotide replaced by RNA.
[e] Fluorescent molecules.

prevalence of the His275Tyr mutation among seasonal H1N1 viruses from the 2007-2008 and the 2008-2009 seasons and H1N1pdm viruses from the 2009-2010 season in Niigata, Japan.

MATERIALS AND METHODS

Sample collection and virus isolation. Nasopharyngeal swab specimens were collected from patients with influenza-like illness who visited a pediatric clinic in Niigata City, Japan, during three influenza seasons (2007-2008 season from January to March in 2008, the 2008-2009 season from January to March in 2009, and the 2009-2010 season in November and December in 2009). Samples were taken after a written informed consent was obtained. None of the patients had received anti-influenza virus drugs before samples were taken. The nasopharyngeal swabs were suspended in viral transport medium and kept at 4°C until transportation to the Division of Public Health, Department of Infectious Disease Control and International Medicine, Niigata University, within 1 week. Initial isolation of influenza viruses was performed using Madin-Darby canine kidney (MDCK) cells. One hundred-microliter aliquots of the supernatants of the nasopharyngeal swabs were inoculated onto MDCK cells, and the cells were then incubated at 34°C with 5% CO_2 until a specific cytopathic effect was detected. Influenza virus isolates were typed and subtyped by hemagglutination inhibition assay using guinea pig red blood cells and commercially available influenza vaccine strain antisera (Denka Seiken Co., Ltd., Tokyo, Japan).

RNA extraction and reverse transcription. Viral RNA was extracted from 100 μl of supernatants of nasopharyngeal swabs or virus culture supernatant using an Extragen II kit (Kainos, Tokyo, Japan), according to the manufacturer's instructions. Reverse transcription was performed using influenza A universal primer Uni12, as reported elsewhere (13). Preparation of RNA from other respiratory viruses was performed using random primers (Invitrogen Corp., Carlsbad, CA) (17).

Primers, probes, and PCR conditions. Two PCR primer pairs were designed to amplify specifically the NA gene of seasonal H1N1 and H1N1pdm viruses (Table 1). Cycling probes for seasonal H1N1 viruses, sH1N1-His275 and sH1N1-Tyr275, were synthesized to detect the SNP at codon ATG/A, which corresponds to CAT (oseltamivir-sensitive His275 genotype) and TAT (oseltamivir-resistant Tyr275 genotype) in the reverse complement (TaKaRa Bio Inc., Japan) (Table 1). Likewise, the cycling probes for pandemic H1N1 viruses, H1N1pdm-His275 and H1N1pdm-Tyr275, were synthesized to detect the SNPs CAC (oseltamivir-sensitive His275 genotype) and TAC (oseltamivir-resistant Tyr275 genotype) (TaKaRa Bio Inc.) (Table 1). The underlined nucleotides indicate the RNA replacement in the chimeric probes used in the real-time PCR. The probes for seasonal H1N1 virus, sH1N1-His275 and sH1N1-Tyr275, were designed in the reverse-complement direction, and the probes for pandemic H1N1 virus, H1N1 pdm-His275 and H1N1 pdm-Tyr275, were designed such that the nucleotide replaced in the RNA sequence is adjacent to the SNP. Cycling probes were labeled with either 6-carboxyfluorescein (FAM) or 6-carboxy-X-rhodamine (ROX), which can detect the oseltamivir-sensitive genotype and the oseltamivir-resistant genotype, respectively.

Cycling probe real-time PCR was carried out using a CycleavePCRCore kit (TaKaRa Bio Inc.). Conditions of the PCR cycles were as follows: initial denaturation at 95°C for 10 s, followed by 40 cycles of denaturation at 95°C for 5 s, primer annealing at 55°C and 59°C for seasonal H1N1 and for H1N1pdm, respectively, for 10 s, and extension and subsequent detection of fluorescence at 72°C for 15 s. In each PCR run, one set of forward and reverse PCR primers and two (FAM- and ROX-labeled) cycling probes were used. Separate PCR runs are needed for seasonal H1N1 and H1N1pdm virus detection.

Human influenza A (H3N2) virus, influenza B virus, and other common human respiratory viruses, such as respiratory syncytial virus, parainfluenza virus, enterovirus, rhinovirus, human metapneumovirus, and adenovirus, were tested with the same cycling probes and primer sets to examine whether cross-reactions occur by the assay. No animal influenza virus strains were tested. All influenza viruses and other viruses used in this study were collected and isolated at the Division of Public Health, Department of Infectious Disease Control and International Medicine, Niigata University, and the Department of Virology, Niigata Prefectural Institute of Public Health and Environmental Sciences.

Control plasmids. Four positive-control plasmids harboring the NA gene insert from a seasonal H1N1 oseltamivir-sensitive strain (sH1N1-OS), a seasonal H1N1 oseltamivir-resistant isolate with the His275Tyr mutation (sH1N1-OR), an H1N1pdm oseltamivir-sensitive strain (H1N1pdm-OS), or an H1N1pdm oseltamivir-resistant virus with the His275Tyr mutation (H1N1pdm-OR) were constructed. NA gene fragments were amplified using the same PCR primers designed in this study. NA gene inserts were cloned using a Mighty TA-cloning kit (TaKaRa Bio Inc.), according to the manufacturer's instructions.

NAI susceptibility assay. Drug susceptibility testing was performed by the 50% inhibitory concentration (IC_{50}) method in order to validate the results of the cycling probe real-time PCR assay (1). The susceptibility to oseltamivir carboxylate (Roche Products, Ltd., Basel, Switzerland) and zanamivir (GlaxoSmith-Kline, Brentford, United Kingdom) was examined by a previously described fluorescence-based NA inhibition assay using methylumbelliferone *N*-acetylneuraminic acid (MUNANA) as the substrate (14).

DNA sequencing. The sequences of selected samples and control viruses used in this study were determined using previously reported primers (5, 26). The NA sequences were edited and assembled using the DNAStar Lasergene 7 program (Bioinformatics Pioneer DNAStar, Inc., WI).

RESULTS

LOD of cycling probe method. Control plasmids were used to determine the limit of detection (LOD) of each primer/probe set. All control plasmids were tested using a 10-fold dilution series from 1×10^1 to 1×10^7 copies (Fig. 1). The range of the threshold cycle (C_T) values of 1×10^1 copies was from 35 to 39, and the range of C_T values of 1×10^7 copies was from 15 to 17. The LOD for each of the four kinds of control plasmids was 10 copies.

Specificity of cycling probe method. The specificity of the cycling probe real-time PCR assay was determined using pre-

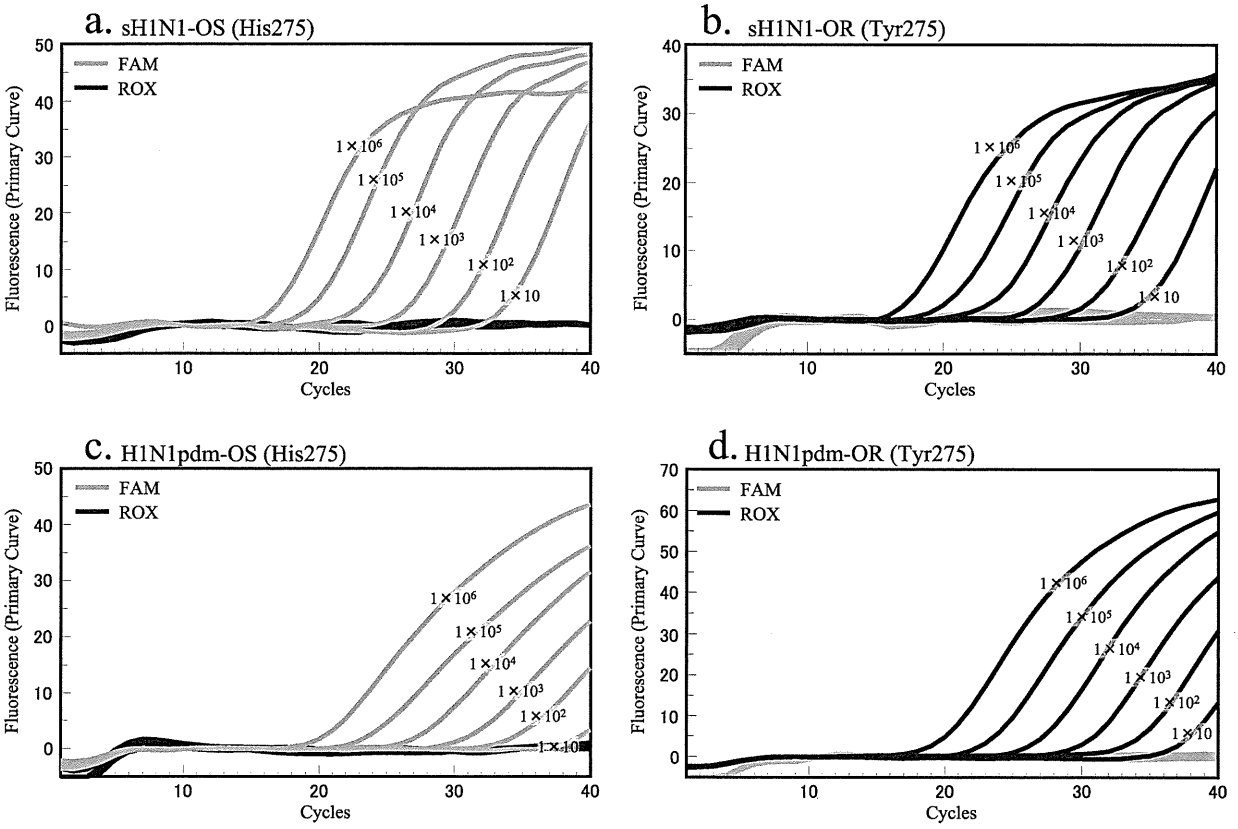

FIG. 1. Limit of detection of cycling probe real-time PCR with control plasmids. FAM fluorescence signals correspond to the oseltamivir-sensitive genotype (His275), and ROX fluorescence signals indicate the oseltamivir-resistant genotype (Tyr275). Control plasmids containing inserts of seasonal H1N1 sequences (sH1N1-OS and sH1N1-OR) reacted with probes sH1N1-His275 and sH1N1-Tyr275, respectively (a and b). Control plasmids harboring H1N1pdm sequences (H1N1pdm-OS and H1N1pdm-OR) reacted with H1N1pdm probes (c and d).

viously characterized seasonal and pandemic H1N1 viruses. Using the seasonal H1N1 primer pair and probe set, all oseltamivir-sensitive seasonal H1N1 nasopharyngeal swabs and isolates tested positive, indicated by the presence of a FAM signal, and all oseltamivir-resistant seasonal H1N1 nasopharyngeal swabs and isolates tested positive, indicated by emission of a ROX fluorescent signal. Importantly, these probes did not show any cross-reactivity with oseltamivir-sensitive H1N1pdm or oseltamivir-resistant H1N1pdm samples (Fig. 2; Table 2). Likewise, when the pandemic H1N1 primers and probes were used, all oseltamivir-sensitive H1N1pdm samples yielded a corresponding FAM signal and all oseltamivir-resistant H1N1pdm samples gave a corresponding ROX signal. The pandemic H1N1 primers and probes did not exhibit cross-reactivity with seasonal H1N1 samples.

The cycling probe method was tested on human influenza A (H3N2) and influenza B viruses and other common respiratory viruses. Results showed that none of these viruses tested positive using the same set of primers and probes (Table 2).

Validation of cycling probe method by NAI susceptibility assay. The median IC_{50}s of oseltamivir carboxylate for oseltamivir-sensitive seasonal H1N1 and H1N1pdm viruses were 2.34 ± 0.70 nM (n = 15) and 2.06 ± 0.99 nM (n = 22), respectively. Oseltamivir-resistant seasonal H1N1 and H1N1pdm viruses exhibited a 300- to 400-fold increase in IC_{50} (982.76 ± 421.47 nM, n = 24) compared to the IC_{50}s of the oseltamivir-sensitive seasonal H1N1 and oseltamivir-sensitive H1N1pdm strains. For zanamivir, the median IC_{50}s were 1.91 ± 0.60 nM, 1.10 ± 1.61 nM, and 0.99 ± 0.49 nM for oseltamivir-sensitive seasonal H1N1, oseltamivir-resistant seasonal H1N1, and oseltamivir-sensitive H1N1pdm viruses, respectively. None of the viruses demonstrated reduced susceptibility to zanamivir.

DNA sequencing. Sequencing results were consistent with the findings from the cycling probe real-time PCR assay and NAI susceptibility test. All oseltamivir-resistant viruses had the His275Tyr mutation in the NA gene.

Prevalence of oseltamivir-resistant influenza viruses. A total of 427 influenza A (H1N1) virus isolates that were collected during three epidemic seasons between January 2008 and December 2009 in Niigata in Japan were screened for the prevalence of the His275Tyr mutation that confers resistance to oseltamivir (Table 3). A nasopharyngeal swab specimen was collected from each patient during the individual's first visit to the medical facility, before any anti-influenza drug was administered. In the 2007-2008 influenza season, none of 72 (0%) seasonal H1N1 isolates were oseltamivir resistant; however, in the 2008-2009 season, all (282 of 282, 100%) of the seasonal H1N1 isolates were oseltamivir resistant. In the 2009-2010 season, seasonal H1N1 viruses were not detected and none of 73 (0%) H1N1pdm isolates were oseltamivir-resistant strains (Table 3).

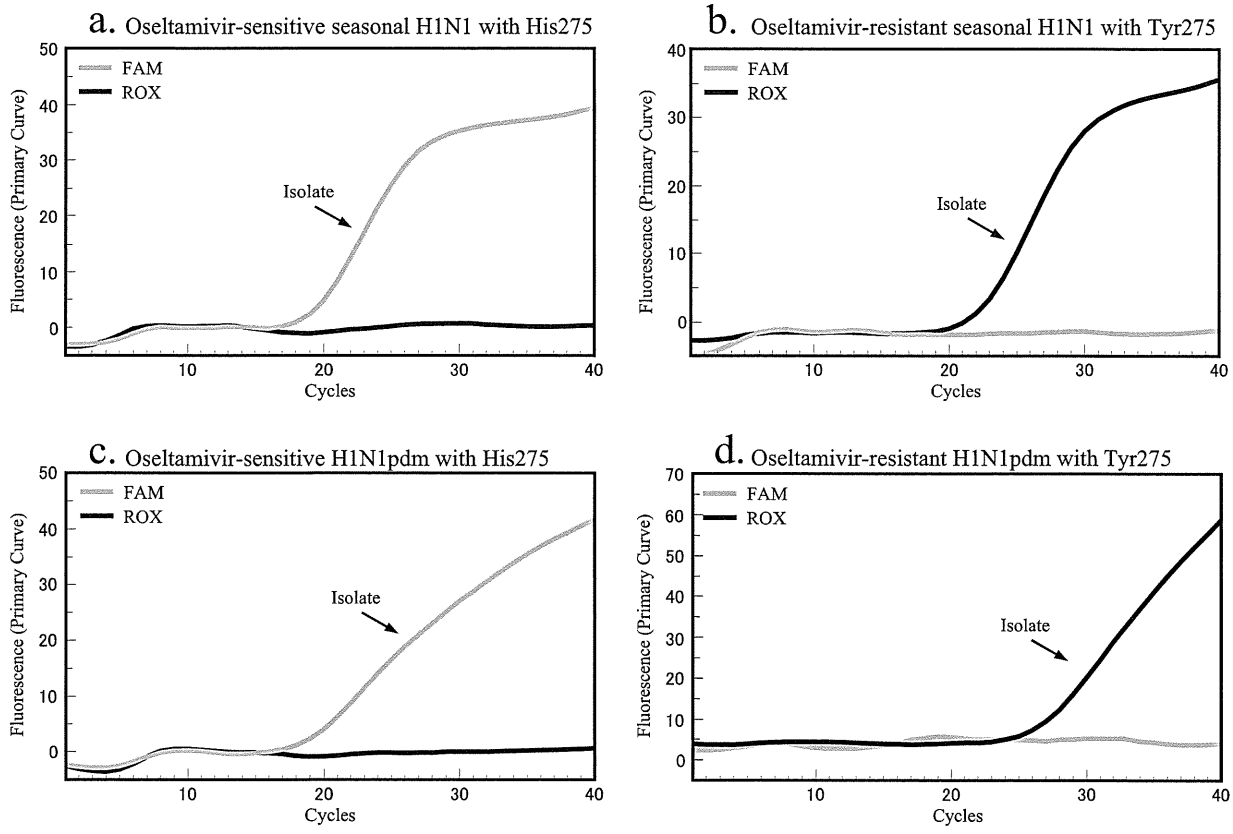

FIG. 2. Detection of oseltamivir-sensitive and -resistant isolates with H275 and H275Y in NA gene of influenza A (H1N1) virus. Oseltamivir-sensitive and -resistant viruses of seasonal H1N1 virus reacted with the FAM probe and the ROX probe, respectively (a and b). Oseltamivir-sensitive and -resistant H1N1pdm viruses reacted with its specific corresponding probes (c and d).

DISCUSSION

This study demonstrated the application of the cycling probe real-time PCR method in detecting the His275Tyr mutation in NA. This method correctly identified the oseltamivir-sensitive (His275) and oseltamivir-resistant (His275Tyr) genotypes of both seasonal and pandemic H1N1 viruses. We previously reported on a cycling probe real-time PCR method for detecting the Ser31Asn mutation in the M2 channel protein which confers resistance to amantadine (19). Our results suggest that the cycling probe real-time PCR method is applicable to detecting drug-resistant viruses by SNP genotyping.

This method showed high specificity in identifying the His275Tyr mutation in NA among human seasonal H1N1 and pandemic H1N1 viruses. The results of this assay were in agreement with the results of the IC_{50} method and gene sequencing. The mutation was detected in both nasopharyngeal swab samples and virus isolates, despite the difference in the virus concentration between the two types of samples. In addition, the method did not show any false-positive reactions with the other influenza A, influenza B, or other respiratory viruses. Thus, our method is very specific, and it is suitable for the detection of the His275Tyr mutation among human influenza A viruses. However, we could not perform this method on classical swine, triple-swine reassortant, or avian influenza viruses because we can handle only human influenza virus strains, as regulated by law. Although the sequence of the amplified NA gene segment in our cycling probe method showed variations compared to the sequences of nonhuman influenza viruses, further study is needed in order to evaluate the specificity of this assay with nonhuman influenza viruses.

Phenotypic assay, such as IC_{50} method, is the "gold standard" for identifying oseltamivir resistance. However, this method is time-consuming because it requires virus culture. Thus, several rapid detection methods were developed, including pyrosequencing, TaqMan probe real-time PCR assay, and RCA, for screening samples for the His275Tyr mutation, which confers resistance to oseltamivir (7, 12, 21, 22, 25). These methods showed high specificities and sensitivities in detecting the drug-resistant influenza virus. Of these methods, pyrosequencing is well-established and provides a definitive identification of the His275Tyr mutation, as well as other novel mutations that are associated with reduced drug susceptibility (6–8). However, not all laboratories can perform pyrosequencing as a routine assay for influenza virus surveillance because the machine and reagents are expensive and the procedures involved are complex. Thus, we developed the cycling probe real-time PCR assay as a low-cost alternative for screening for the His275Tyr mutation. This method has a high specificity and sensitivity in detecting SNPs which are comparable to those of the TaqMan and RCA methods. In addition, the probes that were used in this study can easily be synthesized by various manufacturers, and the cost of

TABLE 2. Probe reaction performance with various virus samples

Sample type	Virus[a]	Subtype[b]	Susceptibility to oseltamivir[c]	No. of samples	No. of samples positive with:			
					Seasonal H1N1 probe set		H1N1pdm probe set	
					sH1N1-His275	sH1N1-Tyr275	H1N1pdm-His275	H1N1pdm-Tyr275
Isolate	Influenza A virus	Seasonal H1N1	Sensitive	10	10	0	0	0
	Influenza A virus	Seasonal H1N1	Resistant	10	0	10	0	0
	Influenza A virus	H1N1pdm	Sensitive	10	0	0	10	0
	Influenza A virus	H1N1pdm	Resistant	3	0	0	0	3
	Influenza A virus	H3N2	NA[d]	10	0	0	0	0
	Influenza B virus	NA	NA	10	0	0	0	0
	Respiratory syncytial virus	NA	NA	5	0	0	0	0
	Parainfluenza virus	NA	NA	1	0	0	0	0
	Enterovirus	NA	NA	1	0	0	0	0
	Rhinovirus	NA	NA	2	0	0	0	0
	Adenovirus	NA	NA	1	0	0	0	0
Nasopharyngeal swab	Influenza A virus	Seasonal H1N1	Sensitive	15	15	0	0	0
	Influenza A virus	Seasonal H1N1	Resistant	15	0	15	0	0
	Influenza A virus	H1N1pdm	Sensitive	15	0	0	15	0
	Influenza A virus	H3N2	NA	15	0	0	0	0
	Influenza B virus	NA	NA	10	0	0	0	0
	Respiratory syncytial virus	NA	NA	5	0	0	0	0
	Human metapneumovirus virus	NA	NA	5	0	0	0	0
	Negative sample	NA	NA	10	0	0	0	0

[a] Viruses were initially detected by virus isolation and PCR using specific primers.
[b] Typed and subtyped by hemagglutinin inhibition assay with vaccine strain antisera and PCR using specific primers.
[c] Resistant strains of both subtypes had a histidine-to-tyrosine change in residue 275 of the NA gene.
[d] NA, not addressed.

reagents is comparable to that of the reagents for the TaqMan method.

We utilized the cycling probe real-time PCR assay in determining the prevalence of the His275Tyr mutation among H1N1 viruses in three influenza seasons in Niigata, Japan. Our results showed that the prevalence of oseltamivir-resistant seasonal H1N1 strains had increased dramatically from the 2007-2008 season (0%) to the 2008-2009 season (100%), as reported in other studies (1, 15, 19, 20). In the 2009-2010 season, all of our H1N1pdm samples were oseltamivir sensitive. This result suggested that the oseltamivir-resistant H1N1pdm virus has not yet gained the genetic fitness to spread like the oseltamivir-resistant seasonal H1N1 viruses in the 2008-2009 season. It was observed that oseltamivir-resistant H1N1pdm viruses emerged after oseltamivir treatment and prophylaxis (2, 3, 24). One possible explanation as to why we were not able to detect oseltamivir-resistant H1N1pdm strains was that we collected samples from patients only prior to antiviral drug treatment. We are aware that during NAI treatment it is important to collect and examine time series samples from immunocompromised patients and from patients who manifested long-term clinical symptoms for monitoring for the emergence of drug-resistant viruses. Our method, which is capable of obtaining results within 3 h after receipt of nasopharyngeal swabs, is applicable in screening of clinical samples for resistance to oseltamivir during antiviral therapy.

One disadvantage of this method is that a new set of primers and probes has to be developed in the event that a novel drug-resistant strain would emerge during treatment with either oseltamivir, zanamivir, peramivir, or laninamivir (9, 11, 18). However, so far, all of the currently circulating oseltamivir-resistant seasonal H1N1 viruses had the His275Tyr mutation (1, 15, 19, 20), and the oseltamivir-resistant H1N1pdm viruses also possessed the same mutation (2, 3, 24). This mutation is very common among the contemporary oseltamivir-resistant viruses that belonged to the N1 group (1, 2, 3, 15, 19,

TABLE 3. Numbers of oseltamivir-sensitive and -resistant strains of influenza A/H1N1 viruses during 2007-2008, 2008-2009, and 2009-2010 seasons in Niigata

Season	Virus subtype	No. of influenza A (H1N1) virus-positive samples[a]	No. of oseltamivir-resistant viruses[b]	Proportion of oseltamivir-resistant viruses (%)
2007-2008	Seasonal influenza A (H1N1)	72	0	0.0
2008-2009	Seasonal influenza A (H1N1)	282	282	100.0
2009-2010	Seasonal influenza A (H1N1)	0	0	
	Influenza A pandemic (H1N1) 2009	73	0	0.0

[a] Samples were collected at a pediatric clinic in Niigata Prefecture in Japan.
[b] Oseltamivir-resistant viruses with His275Tyr mutation in NA.

20, 24). Thus, should these viruses continue to persist in the future, the cycling probe real-time PCR assay can provide a fast, simple, and low-cost alternative for the laboratory-based surveillance of oseltamivir-resistant viruses.

In summary, we developed a highly sensitive and specific method of detecting the His275Tyr mutation in NA among seasonal H1N1 and H1N1pdm viruses by cycling probe real-time PCR assay. We clarified the prevalence of the His275Tyr mutation in three influenza seasons using this method. We demonstrated that the cycling probe method is applicable in monitoring of drug resistance as part of routine influenza virus surveillance work, and this method may provide information useful to clinicians during antiviral therapy.

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ORIGINAL ARTICLE
Myocardial Disease

A National Survey on Myocarditis Associated With the 2009 Influenza A (H1N1) Pandemic in Japan

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with the 2009 Influenza A (H1N1) Pandemic in Japan organized
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Background: An influenza pandemic occurred in 2009 and myocarditis associated with the 2009 influenza A (H1N1) pandemic was reported among hospitalized patients from August 2009.

Methods and Results: The Japanese Circulation Society organized the Clinical Research Committee on Myocarditis Associated with Influenza Pandemic A (H1N1) 2009 and called for a case report on myocarditis for a national survey. The diagnosis of myocarditis was performed using the Guidelines for the Diagnosis and Treatment of Myocarditis (JCS 2009). Fifteen patients were reported to the committee. Fulminant myocarditis developed in 10 patients. Mechanical circulatory support (intra-aortic balloon pumping (IABP) and/or percutaneous cardiopulmonary support (PCPS)) was used on all 10 patients, 8 of whom were rescued. Abnormalities on echocardiography and elevated cardiac enzymes were seen in most of the patients. Myocarditis was found by endomyocardial biopsy in 6 patients. Three patients had complications with pneumonia.

Conclusions: In reality, myocarditis associated with pandemic influenza A (H1N1) seemed to be more common in hospitalized patients, compared with previous seasonal influenza virus outbreaks. To avoid misdiagnosis of acute myocarditis associated with influenza pandemic A (H1N1) 2009, it is essential to determine the characteristic symptoms, signs, and laboratory findings of acute myocarditis during influenza pandemics. Mechanical circulatory support (IABP and/or PCPS) was required to rescue patients with fulminant myocarditis. (*Circ J* 2010; **74:** 2193–2199)

Key Words: Influenza; Myocarditis; Pandemic

Influenza pandemics occur every 10 to 50 years, and the 2009 influenza A (H1N1) pandemic has been spreading worldwide since the first cases were identified in the USA and the United Mexican States in April 2009.[1–6] Most people infected with the 2009 influenza A (H1N1) pandemic recovered without any sequelae, and hospitalizations were quite rare in Japan until June 2009.[4,5] However, some Japanese cases of myocarditis including fulminant myocarditis caused by the 2009 influenza A (H1N1) pandemic began to be reported in hospitalized patients from August 2009.[6] Thus, a Japanese National Survey, to investigate what is going on in the real world around myocarditis associated with the 2009 influenza A (H1N1) pandemic, was initiated by the Japanese Circulation Society.

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Acute myocarditis is a potentially lethal disease, and the etiological agents of viral myocarditis are enteroviruses including coxsackieviruses, adenoviruses, parvoviruses, hepatitis C virus, human immunodeficiency virus, influenza, and others.[7–13] Fulminant myocarditis causes severe hemodynamic dysfunction and requires high-dose catecholamine and mechanical circulatory support. Fulminant myocarditis caused by viral infection is an uncommon type of myocarditis.[7–9] The frequency of myocardial involvement in influenza infection has varied and fulminant myocarditis associated with influenza infection is exceedingly rare as shown by previous papers, although this probability depends on low affinities

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Table 1. Profile, Baseline Disease, Symptoms and Laboratory Findings of 15 Patients With Myocarditis Associated With Pandemic Influenza A (H1N1) 2009 in Japan

Patient No.	Age/sex	Baseline disease	Symptoms of influenza	Cardiac symptom/ onset (day post symptoms of influenza)	Type of myocarditis and complication	ECG findings	CAG	Cardiac enzyme (peak)	UCG
1	6/M	Asthma	Cough, dyspnea, fever (37.5°C)	Dyspnea/day 3	Fulminant myocarditis, viral pneumonia	VF, Torsade de pointes	Not done	CPK (25,244 IU/L)	Diffuse hypokinesis EF 33.5%, effusion
2	11/F	None	Cough, dyspnea, fever (39.8°C)	Shock/day 3	Fulminant myocarditis, shock	ST elevated	Not done	CPK (4,793 IU/L)	Diffuse hypokinesis EF 25%, pericardial effusion
3	24/F	None	Fever, vomiting	Dyspnea, hypotension/day 6	Fulminant myocarditis, alveolar hemorrhage	ST elevated	Normal CAG	CPK (2,681 IU/L)	Diffuse hypokinesis EF 11%, pericardial effusion
4	30/M	None	Fever, dyspnea, chest pain	Chest discomfort/ day 10	Fulminant myocarditis	T inverted	Normal CAG	CPK (971 IU/L)	Diffuse hypokinesis EF 10%, edema of LV wall
5	31/M	None	Cough, arthralgia, fever (39°C)	Orthopnea, hypotension/day 9	Fulminant myocarditis, shock	ST elevated	Normal CAG	CPK (800 IU/L)	Diffuse hypokinesis, edema of inferior LV wall, pericardial effusion
6	31/F	Hyperthyroid	Dyspnea, fever (39°C)	Dyspnea, hypotension/day 2	Fulminant myocarditis, MOF, DIC	SVT, AF	Normal CAG	CPK elevated	Severe hypokinesis and akinesis
7	34/F	None	Vomiting	Chest discomfort/day 1	Fulminant myocarditis	VF, complete AV block	Normal CAG	CPK (3,808 IU/L)	Diffuse hypokinesis EF 32%, LV wall thinning
8	44/M	Asthma	Fever	Dyspnea/day 21	Congestive heart failure	Multifocal PVCs SVT	Normal CAG	No information	Diffuse hypokinesis EF 16%
9	44/F	Asthma	Fever (38.3°C)	Dyspnea, hypotension/day 3	Fulminant myocarditis, shock	VF, frequent VT	Normal CAG	CPK (2,911 IU/L)	Diffuse hypokinesis EF 24%
10	53/M	None	Fever	Syncope/day 3	Complete AV block	Complete AV block	Normal CAG	CPK elevated	Diffuse hypokinesis EF 40%
11	61/M	DM	Nausea, dyspnea, fever (38.8°C)	Dyspnea/day 2	Fulminant myocarditis	AF, T inverted	Normal CAG	Troponin I elevated	Diffuse hypokinesis EF 20%
12	61/F	None	Fever, abdominal pain, disorientation	Dyspnea, hypotension/day 2	Renal failure, septic shock, DIC	ST elevated	Not done	CPK (2,811 IU/L)	Diffuse hypokinesis EF 20%
13	66/M	Emphysema	Common cold-like symptoms	CPA/day 7	CPA, pneumonia (Streptococcus pneumonia)	VF, complete AV block	Normal CAG	CPK (1,842 IU/L)	Almost normal wall motion
14	69/M	Emphysema, cancer, DM	Fever	CPA/day 8	Fulminant myocarditis, cardiac tamponade	Asystole, complete AV block	Normal CAG	CPK elevated	Diffuse hypokinesis EF 29%
15	72/M	IHD (OMI of posterior wall)	Dyspnea, fever	Dyspnea/day 2	Congestive heart failure, viral pneumonia	Giant negative T	No stenosis (s/p PCI)	CPK (827 IU/L)	Diffuse hypokinesis (anterior+posterior wall) EF 38%

Abbreviations see in text.

Table 2. Viral Diagnosis, Treatment and Outcome of 15 Patients With Myocarditis Associated With Pandemic Influenza A (H1N1) 2009 in Japan

Patient No.	Age/ sex	Rapid influenza diagnostic testing	RT-PCR	Ventilator/ days	Mechanical support/days	Other treatment	Biopsy or autopsy	Outcome
1	6/M	Negative (day2,3)	2009A (H1N1) (day 3)	Used/ 14 days	PCPS/11days	Steroid 2 mg/kg, large amount of γ-globulin	Not done	Improved (EF 63%)
2	11/F	Negative (day1) →positive (day3)	Not done	Used/ 22 days	PCPS/3days IABP/4days	Steroid pulse, large amount of γ-globulin	Not done	Improved (EF 70%)
3	24/F	Positive (day1)	Not done	Used	PCPS/7days IABP	Usual dose γ-globulin	Not done	Improved (EF 71%)
4	30/M	Negative (day1) →positive (day2)	Negative (day 20)	Not used	IABP/3days	Not used	Active myocarditis	Improved
5	31/M	Negative (day1)	2009A (H1N1) (day 9)	Not used	IABP/3days	Not used	Myocarditis (mild)	Improved
6	31/F	Positive (day1)	2009A (H1N1) (day 1)	Used/ 15days	PCPS/7days IABP/12days	CHDF, plasmapheresis	Myocarditis	Improved
7	34/F	Negative (day1)	2009A (H1N1) (day1)	Used/ 5 days	PCPS/4days IABP/5days	Plasmapheresis	Not done	Death on day 5
8	44/M	Positive (day2)	Not done (A/CF32X day45)*	Not used	Not used	Not used	Healing myocarditis (day 67)	Incompletely improved (EF 25%)
9	44/F	Negative (day2)	2009A (H1N1) (day 3)	Used	PCPS/6days IABP/12days	Usual dose γ-globulin	Myocarditis (day 3 and 24)	Improved (EF 60%)
10	53/M	Positive (day 1)	2009A (H1N1)	Not used	Not used	Pacemaker	Done	Improved
11	61/M	Positive	2009A (H1N1)	Used	IABP	Not used	Myocarditis (day 4 and 10) negative RT-PCR	Improved
12	61/F	Positive (day1)	Not done	Not used	Not used	Usual dose-globulin	Not done	Improved (EF 50%)
13	66/M	Not done	2009A (H1N1) (day7)	Used/ 4 days	Not used	Not used	Interstitial fibrosis (day 31)	Improved
14	69/M	Positive (day1)	2009A (H1N1) (day1)	Used/ 2 days	PCPS/2days	Not used	Interstitial fibrosis (Autopsy)	Death on day 9
15	72/M	Positive (day2)	2009A (H1N1) (day4)	BiPAP	Not used	Usual dose γ-globulin	Interstitial fibrosis (day 8)	Incompletely improved (EF 44%)

*CF titer to Influenza virus A was 1:32 on day 45.
Abbreviations see in text.

to cardiac involvement of the influenza virus and/or the methods used to diagnose myocardial involvement and influenza infection.[7–9] Although usually both the diagnosis and treatment of the pathogen involved in myocarditis are difficult, good diagnostic methods, such as influenza tests, which were quickly checked and reverse transcription polymerase chain reaction (RT-PCR) for the 2009 influenza A (H1N1) pandemic, and treatment with neuraminidase inhibitors were already available in this 2009 pandemic.[1–6]

Methods

To investigate what is going on in the real world around myocarditis associated with the 2009 influenza A (H1N1) pandemic, the task forces directed by the Japanese Circulation Society organized a Clinical Research Committee for Myocarditis associated with the pandemic. The Japanese Circulation Society called for a case report on myocarditis associated with the 2009 influenza A (H1N1) pandemic to all members (24,203 persons in total) from all regions of Japan through direct e-mail in November 2009. Fifteen cases were immediately reported to the task forces within 4 months, and all cases were analyzed. The diagnosis of myocarditis was performed using the Guidelines for the Diagnosis and Treatment of Myocarditis (JCS 2009).[10] Compatible clinical symptoms, echocardiographic abnormalities in the absence of cardiac ischemia, leakage of cardiac enzymes and/or other evidence of myocardial damage provided the highly probable diagnosis for myocarditis. Myocardial biopsy or autopsy provided the histological diagnosis for myocarditis. Laboratory diagnosis of influenza pandemic A (H1N1) 2009 was made by quick influenza diagnostic testing and/or probe-based RT-PCR using a nasopharyngeal swab or sputum.

Results

Fifteen patients (9 men and 6 women, mean age 42.4±20.8 years) were reported to the task force of the Clinical Research for Myocarditis associated with the 2009 influenza A (H1N1) pandemic. They were admitted to hospitals from August 2009 to February 2010. The profiles, baseline disease, symptoms and laboratory findings of these 15 patients are shown in Table 1, and the results of diagnostic tests for influenza infection, treatment, histological findings and outcome in Table 2. Myocarditis was proven by endomyocardial biopsy in 6 patients, and myocarditis was clinically diagnosed based on clinical figures, leakage of cardiac enzymes, abnormalities on echocardiography, and other findings in the other 9 patients.[10] History was useful in obtaining the correct diagnosis. Thirteen patients complained of typical symptoms of

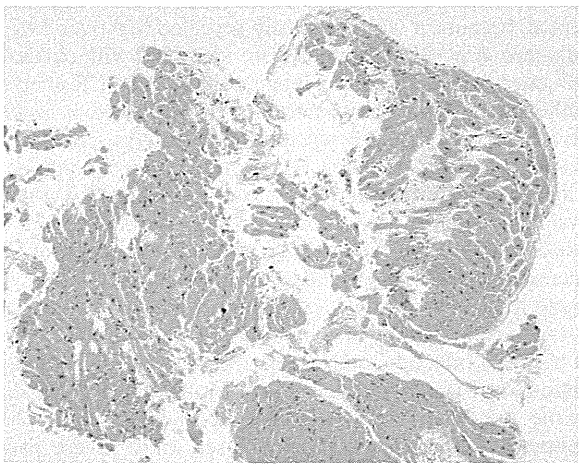

Figure. Endomyocardial biopsy (HE staining) from a 31-year-old woman who developed multi-organ failure (case 6) is shown. She was rescued with mechanical circulatory support (IABP and PCPS). Myocyte degeneration with lymphocyte infiltration and interstitial edema can be seen. IABP, intra-aortic balloon pumping; PCPS, percutaneous cardiopulmonary support.

influenza, such as high fever, cough, headache, and arthralgia. Three patients (20%) complained of abdominal symptoms (abdominal pain, nausea, and vomiting), and one patient complained of common cold-like symptoms. Cardiac symptoms such as dyspnea, chest discomfort, hypotension and syncope developed on the 2nd–21st day of sickness. The most frequent baseline disease was a respiratory disorder in 5 (33%) patients (bronchial asthma in 3 patients and emphysema in 2 patients), and others were diabetes in 1 patient and ischemic heart disease in 1 patient. Three patients with myocarditis were complicated by pneumonia: viral pneumonia in 2 patients and bacterial pneumonia in 1 patient.

Most patients exhibited electrocardiogram (ECG) abnormalities, including ventricular fibrillation in 3 patients, complete AV block in 4 patients, ST elevation in 4 patients, giant negative T-waves in 1 patient, and atrial fibrillation in 1 patient. Two patients were emergently admitted because of cardiopulmonary arrest due to ventricular fibrillation or complete AV block. Echocardiography revealed abnormalities of left ventricular wall motion in 14 patients and pericardial effusion in 2 patients. Cardiac dysfunction almost completely recovered in 11 patients but only incompletely in 2 patients. Coronary angiography was performed on 12 patients, which yielded normal results in all. Cardiac enzymes were elevated in 14 patients, with a peak serum creatine kinase concentration of 800 to 25,244 (IU/L). Quantitative troponin testing was measured in 4 patients and was found to be elevated in all patients. Qualitative quick troponin testing was measured in 3 patients, and was positive in 1 patient.

The clinical manifestations of myocarditis caused by influenza pandemic A (H1N1) varied greatly, and 10 patients were diagnosed with fulminant myocarditis with fatal arrhythmias, and/or varying degrees of cardiogenic shock. Cardiopulmonary arrest was the first cardiac symptom in 2 patients. Syncope due to complete AV block was the first cardiac symptom in 1 patient, who was rescued with temporary pacemaker implantation. The clinical course of patients also varied. Cardiac dysfunction progressed rapidly in 12 patients; 10 of these (83%) recovered to their previous condition and 2 died. Cardiac dysfunction developed after recovery from flu-like symptoms in 2 patients, and myocarditis appeared to have persisted for 1.5 months in 1 patient.

Quick diagnostic testing for influenza, performed on the first visit to the clinic, was negative in 5 patients and positive in 8 patients.[4–6] Quick diagnostic testing, when performed a second time was positive in 2 patients who initially had negative results. RT-PCR testing for the 2009 influenza A (H1N1) pandemic yielded positive results in 10 patients. RT-PCR or quick diagnostic testing yielded positive results in all patients.

Endomyocardial biopsy was performed in 8 patients and an autopsy was performed in 1 patient. Endomyocardial biopsies demonstrated histological myocarditis in 6 patients. Histological findings of an endomyocardial biopsy from case 6, who developed multi-organ failure, are shown in Figure. She was rescued with mechanical circulatory support. Lymphocyte infiltration and interstitial edema could be seen. RT-PCR testing for pandemic influenza A (H1N1) from a biopsy specimen was performed only in 1 case, but it was negative.

A ventilator was used in 6 patients and a biphasic positive airway pressure support system (BIPAP) was used in 1 patient. Mechanical circulatory support with intra-aortic balloon pumping (IABP) and/or percutaneous cardiopulmonary support (PCPS) was emergently inserted in 9 patients. Seven patients were rescued with mechanical circulatory support, and 2 patients died. A temporary pacemaker was implanted into the patient with complete AV block. All patients were treated with neuraminidase inhibitors, and high-dose immunoglobulin was used in 2 patients. Corticosteroid was used only in 2 children, but not in any adult patients.

Discussion

The influenza pandemic began in Kobe and Osaka, the middle region of Japan, in May 2009, and all patients recovered until July 2009 without any sequelae.[4,5] It was reported that, in Canada, critical illness due to the 2009 influenza A (H1N1) pandemic occurred rapidly after hospital admission, often in young adults, and was associated with severe hypoxemia, multisystem organ failure, which necessitated prolonged mechanical ventilation, and the frequent use of rescue therapy.[1–3] Hospitalization and death caused by the 2009 influenza A (H1N1) pandemic increased from August 2009, but this was rare in Japan. The Ministry of Health, Labor and Welfare (MHLW) of Japan confirmed only 85 deaths by 1st December 1, 2009, although the estimated number of cases was about 12.6 million by the end of November.[6] Japanese data obtained from the MHLW website showed that the proportion of influenza-like illness cases in those aged 0–4 years and in adults was lower in Japan, compared with other countries. More than 75% of cases were those aged 5–19 years with a low rate of fatalities in Japan. The mortality rates, both per reported rates and per hospitalizations, increased significantly with age. The mean age of the 15 myocarditis cases associated with the 2009 influenza A (H1N1) pandemic was 42 years, which was higher than the mean of estimated cases and similar to other fatal cases during the pandemic in Japan.[6]

Acute myocarditis is a potentially lethal disease, and the etiological agents of viral myocarditis are enteroviruses including coxsackieviruses, adenoviruses, parvoviruses, hepatitis C virus, human immunodeficiency virus, influenza, and others.[7–10] Coxsackievirus B has been described as the most common pathogen of viral myocarditis, and hepatitis C virus is associated with many different forms of heart disease world-

wide, however, influenza myocarditis is relatively rare.[11–16] Bratinssak et al reported four fulminant myocarditis cases in patients aged from 3 to 9 associated with the 2009 influenza A (H1N1) pandemic within a 30-day period, and this suggested that the 2009 influenza A (H1N1) pandemic was more commonly associated with myocarditis than seasonal influenza.[14] Martin et al identified 6 patients with reversible cardiac dysfunction associated with pandemic influenza A (H1N1) out of 123 hospitalized pandemic influenza A (H1N1) patients.[15] There was only 1 case report of fulminant myocarditis with the 2009 influenza A (H1N1) pandemic in Europe.[16] We report herein 15 myocarditis patients varying in age from a child to an old man over 70-year-old as a result of cross-sectional national survey by assist from all members of the Japanese Circulation Society using direct e-mailing system. We suggest that myocarditis is, along with pneumonia and encephalopathy, an important cause of clinical deterioration in influenza patients in Japan. Myocarditis associated with the 2009 influenza A (H1N1) pandemic seemed to be more common in hospitalized patients, compared with previous seasonal influenza virus.[6]

The diagnosis of myocarditis was performed using the Guidelines for the Diagnosis and Treatment of Myocarditis (JCS 2009) with a hybrid of compatible clinical symptoms, evidence of cardiac dysfunction, abnormality of cardiac enzymes in the absence of active coronary ischemia or other evidence of myocardial damage.[10] Clinical symptoms of these patients were not specific; however, most patients complained of not only upper respiratory symptoms but also systemic symptoms. ST elevation was seen in 4 patients and giant negative T-wave was seen in 1 patient, so ECG findings were not specific. Echocardiography revealed reversible abnormalities of left ventricular wall motion, and cardiac enzymes were elevated in most patients. Quantitative troponin was measured in 4 patients and elevated in all; however, qualitative quick troponin testing was measured in 3 patients, but was positive in only 1 patient in this study. Reichlin et al reported that sensitive cardiac troponin assays improve the early diagnosis of myocardial infarction, so we recommend that these assays might be useful for the diagnosis and management of myocarditis.[17] Cardiac scintigram is also useful.[10,18,19] A new approach to diagnose myocarditis is cardiovascular magnetic resonance (CMR) imaging.[10] Liu and Yan observed that CMR imaging is helpful for the detection of myocarditis, because CMR can visualize the entire myocardium.[18] CMR probably has good sensitivity in detecting patchy processes and changes in tissue composition associated with inflammation. To avoid misdiagnosis of acute myocarditis as a complication of influenza infection, it is essential to determine the characteristic symptoms, signs, and laboratory findings of acute myocarditis during influenza infection. Myocarditis is probably underdiagnosed, so we have to strongly suspect myocarditis in hospitalized patients during an influenza pandemic.

Influenza is an acute respiratory illness caused by infection with influenza viruses. The most frequent baseline disease in the present study was lung disease, in 5 (33%) patients (asthma in 3 patients and emphysema in 2 patients). RT-PCR assays or quick diagnostic testing yielded positive results in 15 patients, and viral pneumonia were complicated in 2 patients in this study. Nasopharyngeal smears or sputum were positive for influenza pandemic A (H1N1) 2009 virus on RT-PCR assay in 10 patients. RT-PCR assay was not performed or was negative, and further characterization of the virus was not performed in the other 5 patients. However, pandemic influenza A (H1N1) infection was strongly suspected, because it was previously revealed that over 99% of influenza A positive samples were identified with pandemic influenza A (H1N1) during this period by RT-PCR analysis. Although quick diagnostic testing for influenza is usually performed in Japan, the sensitivity of this type of testing is not high enough.[4–6] Quick diagnostic testing for influenza, which was performed at the first visit to the clinic, was negative in 5 patients. One child who was hospitalized in the Osaka Medical College Hospital under the diagnosis of viral pneumonia caused by pandemic influenza A (H1N1) was found to be positive for viral pneumonia based on the chest X-ray, accumulated white blood cell counts in the absence of bacterial infection, high serum C-reactive protein concentrations and negative quick diagnostic testing for influenza on the 2nd day of sickness. Itoh et al reported that the 2009 influenza A (H1N1) pandemic caused more severe pathological lesions in the lungs of infected mice, ferrets, and non-human primates than the seasonal human H1N1 virus.[20] Nakajima et al reported that the concentrations of various cytokines/chemokines in the serum and autopsied lung tissue were elevated in both of the first autopsy cases in Japan.[21] Muneuchi et al reported that myocarditis associated with influenza B virus appeared to be caused by endothelial impairment and disturbance of microcirculation rather than direct injury to cardiac myocytes.[22] These findings suggest that a negative quick diagnostic test of patients with systemic symptoms might lead to hospitalization due to symptomatic viral pneumonia, or myocarditis, and the pathogenesis of systemic complications of influenza might be related to the induction of inflammatory cytokines produced by infected alveolar cells.

The clinical manifestations of myocarditis quite varied. Cardiac dysfunction progressed rapidly in 12 patients, and cardiac dysfunction developed after recovery from flu-like symptoms in 2 patients, and probably persisted for 1–1.5 months in 1 patient. Cases including slow progressive myocarditis and repetitive myocarditis are very rare. Takehana et al reported that a 75-year-old man who recovered from myocarditis associated with influenza A developed cardiogenic shock and died of fulminant myocarditis.[23] Most patients who survived recovered without any cardiac sequelae in this study, quite similar to previous reports. The degree of myocarditis associated with the 2009 influenza A (H1N1) pandemic was in the present study relatively mild even in patients with fulminant myocarditis.[24] Kotaka et al reported that murine influenza myocarditis was histologically mild and brief in duration compared to coxsackievirus B3 myocarditis.[25] We suggest that the pathogenesis and pathomechanism of pandemic influenza myocarditis differ depending on the pathogen,[25] and moreover, that significant mechanisms of cardiac injury, such as cytokine storm, endothelial dysfunction, oxidative stress and other factors, might play significant roles in the pathogenesis of pandemic influenza myocarditis.[21–27] RT-PCR testing for pandemic influenza A (H1N1) from biopsy specimens was performed in only 1 case, and was negative. To evaluate influenza viral persistence in the myocardium in cases with myocarditis, further evaluation of viral replication in the myocardium using RT-PCR or in situ hybridization methods for pandemic influenza A (H1N1) from myocardial specimens is inevitable.

The first therapy for myocarditis patients with heart failure is supportive intervention. The recent application of PCPS and/or IABP to serious cases of viral myocarditis has yielded good outcomes.[5,29] The severity and grade of cardiac and renal

dysfunction are important factors in connection with the prognosis. In this study, 8 patients were rescued using mechanical circulatory support and 2 patients died. It is important to recognize that patients with influenza infection might have acute myocarditis with heart failure, and that early diagnosis is required for adequate treatment.

Neuraminidase inhibitors work by blocking the function of the viral neuraminidase protein and thus prevent the virus from reproducing by budding from the host cell, and are useful for treating and preventing influenza virus infections.[1–6,20] Itoh et al reported that the 2009 influenza A (H1N1) pandemic is sensitive to neuraminidase inhibitors, suggesting that these drugs could function as a first line of defense against the 2009 influenza A (H1N1) pandemic.[1–6,20] Treatment with neuraminidase is also recommended by the Japanese Association of Infection. The low rate of case fatality in Japan could be a result of aggressive early intervention using with antiviral drugs such as oseltamivir and zanamivir. All of the present patients were treated with oseltamivir. In Japan, 3 types of neuraminidase inhibitors are available: oseltamivir, zanamivir, and peramivir. Adamantine, a M2 protein blocker, is resistant to the 2009 influenza A (H1N1) pandemic, while oseltamivir and zanamivir exhibit lower frequencies of antiviral resistance.[29] Oseltamivir is orally available, while zanamivir is inhaled.[25] Halvala et al identified 10 patients who developed the H275Y oseltamivir-resistance mutation out of 1,802 samples in Scotland.[30] The new neuraminidase inhibitor, peramivir, is formulated for intravenous administration and it has resulted in a good IC_{50} response for pandemic influenza virus. Peramivir is recommended for patients requiring ventilation with difficulty in absorption of oseltamivir.[29] Treatment with high-dose immunoglobulin was used in 2 patients, which is still controversial.[31] Corticosteroid was used only in 2 children, but not in adult patients. We know that inflammatory cytokines are important in the infection course of influenza; however, immunosuppression therapy with corticosteroids is not recommended, and this is supported by previous evidence.[2,10] Moderate- to high-dose steroids are not recommended by the World Health Organization, because they are of unproven benefit and potentially harmful.

Conclusion

As a result of the present national survey in Japan, myocarditis associated with the 2009 influenza A (H1N1) pandemic seemed to be more common, compared with previous seasonal influenza viruses. To avoid misdiagnosis of acute myocarditis caused by influenza pandemic A (H1N1) 2009, the characteristic symptoms, signs, and laboratory findings of acute myocarditis during influenza pandemics must be determined. Mechanical circulatory support (IABP and or PCPS) was required to rescue myocarditis patients by all means. Appropriate treatment using neuraminidase inhibitors must be recommended for patients with myocarditis associated with the influenza A (H1N1) pandemic.

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Appendix

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ORIGINAL ARTICLE

Comparison of the clinical symptoms and the effectiveness of neuraminidase inhibitors for patients with pandemic influenza H1N1 2009 or seasonal H1N1 influenza in the 2007–2008 and 2008–2009 seasons

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Abstract The clinical symptoms and effectiveness of neuraminidase inhibitors (NAI) have not been adequately compared among pandemic H1N1 2009 patients, seasonal H1N1 patients, and patients with H1N1 with the H275Y mutation. The data of 68 seasonal H1N1 patients in 2007–2008, 193 seasonal H1N1 patients in 2008–2009, and 361 pandemic H1N1 2009 patients diagnosed by PCR who received an NAI were analyzed. The duration of fever (body temperature ≥37.5°C) after the first dose of NAI and from onset was calculated. The H275Y neuraminidase mutation status was determined for 166 patients. Significantly lower mean age (18.4 ± 13.2 years) and a higher percentage of teenagers (53.7%) were found for pandemic 2009 influenza than for seasonal influenza ($P < 0.001$). The peak body temperature was equivalent (mean, 39.0°C) in the three seasons, and the frequency of symptoms was the same or lower for pandemic influenza compared with seasonal H1N1. None of the 34 analyzed pandemic H1N1 virus isolates contained the H275Y mutation, which was commonly detected in the 2008–2009 season. The duration of fever after the start of oseltamivir therapy was significantly shorter for patients with pandemic (23.0 ± 11.6 h) than with seasonal H1N1 in both the 2008–2009 (49.7 ± 32.3 h) and 2007–2008 seasons (32.0 ± 18.9 h). The mean duration of fever after the first dose of zanamivir was not different among the three seasons (26.9–31.5 h). Clinical symptoms were the same or somewhat milder, and oseltamivir was more effective, for pandemic 2009 than for seasonal H1N1 influenza with or without H275Y mutation.

Keywords Oseltamivir · Zanamivir · Pandemic influenza · H1N1 · Seasonal influenza · H275Y mutation

Introduction

The H3N2 influenza A virus was prevalent in Japan for 10 years before the 2007–2008 season; however, the H1N1 virus became the most prevalent in the 2007–2008 and 2008–2009 seasons. The H275Y mutation in the neuraminidase that confers oseltamivir resistance was rarely seen in 2007–2008 but was common in the 2008–2009 season [1–3]. In our previous study, H1N1 with the H275Y mutation showed an in vitro reduction in susceptibility to oseltamivir of approximately 1/200. The clinical effectiveness of oseltamivir, but not zanamivir, estimated by body temperature and viral persistence, decreased significantly for seasonal H1N1 virus with the H275Y mutation in the 2008–2009 season compared to that without the H275Y mutation in the 2007–2008 season, especially in children [1, 2].

Since May 2009, the pandemic H1N1 2009 virus has spread throughout Japan [4]. Studies of the clinical symptoms of the pandemic 2009 virus and the effectiveness of

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