医療機関にかかることができた。また，提供される医療レベルも高く，医療機関側の感染防止対策も整備されている。わが国では，世界的には使用習慣の少ないマスクの着用や石鹸での手洗いなど，感染伝播を抑える生活習慣もあり，公衆衛生に対する意識が高い。言い換えれば，既に早期診断・早期治療体制，感染拡大防止体制が整っている。これらの社会的背景が，わが国の重症例を少なくしている重要な要因になっていると思われる。

これらのメキシコ，日本の事情から理解されると思うが，グローバルな感染症といえども，社会的・経済的・医療体制のあり方がその発生と社会における疾患重篤性に強く影響している。つまり，インフルエンザはグローバルな疾患であるが，重篤性はウイルスの特性のみに規定されるものでなく，一国の社会・経済的事情や医療制度に強く規定されていることが示唆される。これらのことから，インフルエンザの重症化を防止するには，医療インフラを整備する政策と，国民への疾病に対する理解の徹底，正確な情報の迅速な提供が不可欠であるといえよう。

われわれの共同研究先であるメキシコ国立呼吸器疾患センター(Instituto Nacional de Enfermedades Respiratorias)は第3次高度医療機関であるため，軽症の患者は通常ほとんど診療しない。感染症の外来患者は1日平均約15人，入院患者は5人程度であった。パンデミック(H1N1)2009の発生以降，2009年4月末には1日の外来が300人を超える日もあり，HIV/AIDS病棟など他疾患病棟を閉鎖し，本疾患の対応に当たった。世界保健機関(WHO)の宣言と同日の4月23日，メキシコ保健大臣によって新型インフルエンザの発生と，春休みから戻ったばかりであった学校を1週間休校にすること，抗ウイルス薬による無料治療プランがあることが発表された。これは，その後のメキシコのパンデミック(H1N1)2009の症例数と重症・重篤・死亡例の減少に大きく貢献することとなったと思われる。つまり，学校閉鎖という政策による感染拡大の防止，国民への早期治療の重要性の喚起，国民の医療アクセスの容易化，抗ウイルス薬の早期投与が可能になったのである。

おわりに

インフルエンザ重症化の防止と治療には，医学的見地からの対応だけでは十分であるとはいえない。今後のインフルエンザパンデミックの臨床対応と診療体制の検討には，医学的，社会・経済的要因，政策などの包括的視点と協調が必要である。疾病の重症化を防止し，有効な治療方法を検討することで，古い時代から続く疾患“インフルエンザ”に対峙したいと考える。

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Rare Influenza A (H3N2) Variants with Reduced Sensitivity to Antiviral Drugs

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In 2007 and 2008 in Myanmar, we detected influenza viruses A (H3N2) that exhibited reduced sensitivity to both zanamivir and amantadine. These rare and naturally occurring viruses harbored a novel Q136K mutation in neuraminidase and S31N mutation in M2.

Adamantanes and neuraminidase inhibitors (NAIs) are the 2 classes of drugs indicated for preventing or treating influenza virus infection. In 2005, the high prevalence of influenza viruses A (H3N2) with S31N mutation in M2 limited the effectiveness of amantadine (*1*,*2*). In 2008, the emergence of subtype H1N1 with H274Y mutation in neuraminidase (NA) raised concerns about the use of oseltamivir (*3*,*4*). On the other hand, the incidence of zanamivir-resistant viruses was low (*5*). In 1998, 1 case of zanamivir-resistant influenza B virus, which was isolated from an immunocompromised child who underwent prolonged zanamivir treatment, was reported (*6*). In 2008, subtype H3N2 with D151A/V mutations in NA demonstrated reduced zanamivir sensitivity by chemiluminescent NAI assay (*5*). Recently, zanamivir-resistant subtype H1N1 isolates with a novel Q136K mutation in NA were isolated in Oceania and Southeast Asia (*7*).

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We report the detection of influenza viruses A (H3N2) harboring a Q136K mutation in NA and an S31N mutation in M2, which respectively confer reductions in zanamivir and amantadine susceptibility. In 2007 and 2008, we performed phenotypic and genotypic analyses in characterizing these viruses from Myanmar.

The Study

Nasopharyngeal swabs were collected from patients with influenza-like illness at Sanpya Hospital in Yangon, Myanmar, and outpatient clinics affiliated with the Department of Medical Research (Central Myanmar) in Nay Pyi Taw. Rapid test kit–positive samples were sent to Niigata University, Japan, for subsequent analyses. Virus isolation and subtyping PCR were performed as previously described (*8*). The NAI susceptibility test was performed by a fluorescence-based NA activity assay that measures the 50% inhibitory concentration (IC_{50}) by using zanamivir and oseltamivir carboxylate (*9*). All samples were assayed in duplicates in ≥2 independent experiments. A sample was considered an extreme outlier if its IC_{50} value was 10× higher than the mean values for sensitive strains with >3 interquartile range from the 25th and 75th percentiles in the box-and-whisker plot analysis (*9*). So far, all known NAI-resistant viruses are extreme outliers (*10*). Screening for S31N mutation in M2 was done by cycling probe real-time PCR (*11*). Sequencing and phylogenetic analysis of the hemagglutinin (HA) and NA genes were performed as previously described (*8*).

A total of 253 and 802 rapid test kit–positive samples were collected in Myanmar in 2007 and 2008, respectively. Of these, 64 isolates of subtype H3N2 were detected in 2007 and 211 in 2008. NAI susceptibility assay showed 1 (1.5%) isolate (A/Myanmar/M187/2007) with a zanamivir IC_{50} value of 59.72 nM, which was collected in August 2007, and 1 (0.5%) isolate (A/Myanmar/M114/2008) with a zanamivir IC_{50} of 33.37 nM, which was collected in July 2008. These isolates respectively demonstrated a 53× and 30× reduction in zanamivir susceptibility (Table) and were extreme outliers (data not shown). On the basis of cycling probe real-time PCR assay, these viruses had an S31N mutation in M2, which confers resistance to amantadine. All subtype H3N2 viruses analyzed in this study remain sensitive to oseltamivir carboxylate (Table).

Phylogenetic analysis of the HA and NA genes showed that the isolates with reduced sensitivity to zanamivir belonged to 2 distinct clusters (Figure 1). These viruses accumulated 2 and 3 amino acid (aa) substitutions in HA and 6 and 2 aa changes in NA in 2007 and 2008 (Figure 1), respectively. Epidemiologic and sequencing data did not suggest any link between the cases. Analysis of the NA

[1]These authors contributed equally to this article.

Table. Characteristics of subtype H3N2 influenza viruses with Q136K mutation in NA and S31N substitution in M2*

Strains	Passage history	NA mutation	IC_{50}s of NA inhibitors: Zanamivir, nM ± SD	Fold change	Oseltamivir, nM ± SD	Fold change	Amantadine sensitivity† (M2 mutation)
All NAI-sensitive subtype H3N2 isolates‡	MDCK2	None	1.12 ± 0.40	1	0.86 ± 0.44	1	Resistant (S31N)
A/Myanmar/M187/2007	MDCK2	Q136K	59.72 ± 3.83	53.3	0.13 ± 0.05	0.2	Resistant (S31N)
A/Myanmar/M114/2008	MDCK2	Q136K	33.37 ± 7.02	29.8	0.16 ± 0.03	0.2	Resistant (S31N)
A/Texas/131/2002§		None	1.43 ± 0.09	1.3	0.99 ± 0.09	1.2	Sensitive
A/Texas/131/2002_E119V§		E119V	5.43 ± 0.68	4.8	94.33 ± 2.06	109.7	Sensitive

*NA, neuraminidase; IC_{50}, inhibitory concentration; NAI, neuraminidase inhibitors.
†Amantadine sensitivity was based on M2 genotyping data.
‡Average IC_{50} was calculated excluding the control viruses (n = 47).
§Reference strains used as drug-sensitive and -resistant control viruses in the NAI assay.

gene showed that the isolates with reduced sensitivity to zanamivir had a glutamine (Q) to lysine (K) substitution at aa position 136. Sequence chromatograms showed a heterogeneous population of virus possessing either Q or K at position 136, with a dominant peak for the K136 mutant (Figure 2). Direct sequencing of primary samples showed a similar profile of chromatogram with a higher signal for the K136 mutant and a minor peak for the Q136 wild-type strain (Figure 2). The rest of the zanamivir-sensitive isolates in 2007 and 2008 had the Q136 genotype, and no NAI-resistant-associated mutations were detected elsewhere in the NA gene.

Conclusions

In this study, we detected a novel influenza virus A (H3N2) with Q136K mutation in NA and S31N mutation in M2, which demonstrated reduced susceptibility to both zanamivir and amantadine but remained susceptible to oseltamivir. These Q136K viruses were isolated at a low frequency (<1.5%) in Myanmar in 2007 and 2008. Phylogenetic analysis showed that these viruses were already amantadine-resistant with S31N mutation in M2. Amantadine-resistant viruses with S31N mutation have been the predominant circulating strains among subtype H3N2 viruses in Myanmar since 2005 (*8*). The Q136K substitution in NA was probably generated by spontaneous point mutation. The HA and NA gene sequences of Q136K mutants

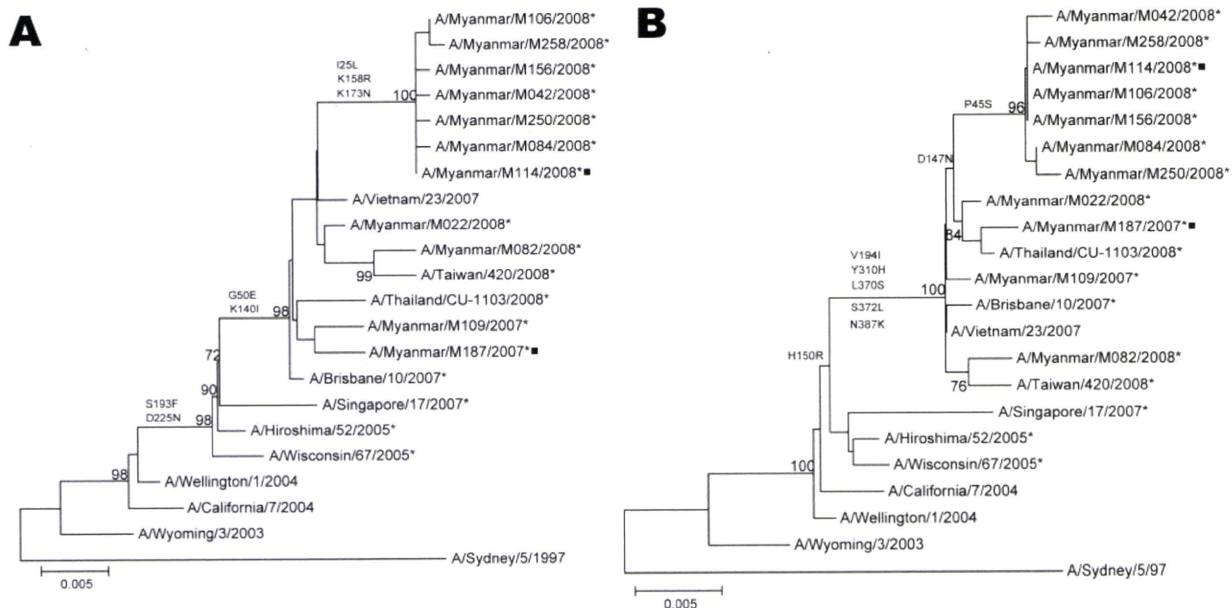

Figure 1. Phylogenetic analysis of the A) hemagglutinin (HA) and B) neuraminidase (NA) genes of influenza virus A (H3N2) isolates in Myanmar in 2007 and 2008. Trees were generated by using the neighbor-joining method. Bootstrap values >70% of 1,000 replicates and amino acid changes that characterize a branch are indicated on the left side of the node. Amantadine-resistant isolates with S31N mutation in M2 are marked with asterisks, and isolates with reduced sensitivity to zanamivir with Q136K mutation in NA are marked with squares. GenBank accession no. of the genomic sequences of isolates are GQ478849–GQ478866. Nucleotide sequences of the HA and NA genes of vaccine strains and isolates from other countries were obtained from the National Center for Biotechnology Information Influenza Virus Resource (www.ncbi.nlm.nih.gov/genomes/FLU). Scale bar indicates nucleotide substitutions per site.

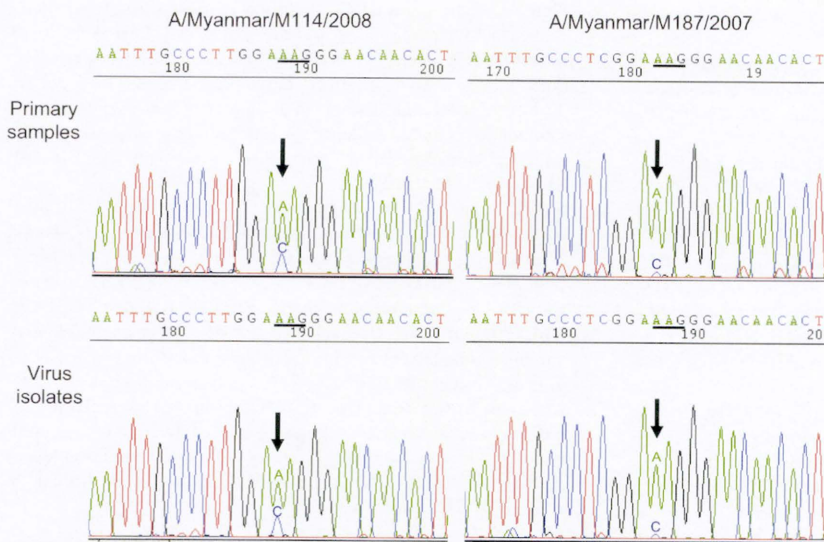

Figure 2. Detection of Q136K substitution in neuraminidase by sequencing in primary samples and virus isolates. Arrows indicate the first peak of the codon encoding amino acid position 136. Comparison of the sequence chromatogram showed a mixed population of bases in both original clinical samples and virus isolates, with a dominant peak for 136K (AAG) mutants, compared with wild-type 136Q (CAG) viruses.

were submitted to GenBank under accession nos. A/Myanmar/M187/2007: FJ229893 (HA), FJ229860 (NA) and A/Myanmar/M114/2008: GQ478854 (HA), GQ478863 (NA).

Hurt et al. recently reported the characterization of zanamivir-resistant subtype H1N1 with Q136K mutation in NA (*7*). Zanamivir IC_{50}s of these viruses ranged from 6 nM to 238 nM (*7*); which differed from the 1–60 nM range of subtype H3N2 viruses obtained in this study. This finding may be due to differences in subtype and variations in the assay. The Q136K mutation was not detected in the primary clinical samples by sequencing (*7*); however, in our study, the Q136K mutation in subtype H3N2 isolates was detected in primary samples. Comparison of the sequence chromatograms between original samples and virus isolates showed a similar profile, suggesting that the Q136K mutants were present in primary samples of subtype H3N2 isolates. The presence of Q136K variants in primary samples appears to be subtype-specific because these mutants were present in very low proportions among subtype H1N1 viruses (*12*). To determine whether mutations exist in other gene segments associated with Q136K mutations, we performed a full genome analysis of Q136K mutants and wild-type viruses. We found no additional mutations in Q136K strains, which suggest that the genetic background of these viruses can compensate for the K136 mutation. However, further study is needed to confirm whether the accumulated 5 aa changes in HA and 8 substitutions in NA would compensate for the Q136K mutation.

We searched the database for NA sequences of influenza viruses A (H3N2) with Q136K mutation that are available on GenBank. Of the 3,381 sequences obtained, 4 sequences from human influenza, which were isolated in 1995, 2003, 2004, and 2007, and 1 sequence from swine influenza, which was isolated in Japan in 1997, contained the Q136K substitution. Sequences from Q136K mutants isolated before 2007 showed no mutations in the M2 gene. The data indicate that these viruses occur naturally because some of the isolates in the database were obtained before introduction of zanamivir into clinical practice in 1999 in Australia, New Zealand, United States, and Europe (*9,13*). In addition, Myanmar patients who shed these Q136K viruses did not receive any NAIs. The clinical relevance of Q136K mutants is unknown. Further study is needed to evaluate the effectiveness of zanamivir in patients infected with Q136K mutants.

Continued monitoring of viruses with reduced sensitivity to NAI and adamantanes is needed, and routine surveillance should include both phenotypic and genotypic assays. The Q136K substitution in NA should be used as a molecular marker associated with reduced NAI susceptibility not only in subtype H1N1 isolates but also among subtype H3N2 isolates.

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etymologia

Yersinia

[yər-sin′-e-ə]

This genus of gram-negative bacteria was named after bacteriologist Alexandre-Émile-John Yersin (1863–1943). Born in Switzerland, he studied medicine in Paris and began a successful early career in the laboratory. He worked on rabies with Pierre Roux and on the tubercle bacillus under Robert Koch in Germany. He later worked at the Institut Pasteur on the toxic properties of the diphtheria bacillus and eventually signed on as a doctor on a ship headed for Saigon and Manila. In 1894, while he still worked for a French shipping company, he investigated an outbreak of plague in Hong Kong. After 7 days in a makeshift laboratory, he isolated the plague bacterium, which he called *Pasteurella pestis*.

Japanese bacteriologist Shibasaburo Kitasato had arrived in Hong Kong, a few days before Yersin and also had isolated the bacterium. Kitasato published his findings in English and Japanese. Yersin published his in French. He also established a laboratory in Nha Trang, Vietnam, where he developed an antiplague serum that reduced the death rate from 90% to ≈7%. Since 1970, the organism has been called *Yersinia pestis*.

Source: Burns W. Alexandre Yersin and his adventures in Vietnam. 2003; Medical Research Council National Institute for Medical Research. http://www.himr.mrc.ac.uk/millhillessays/2003/yersin/; http://www.whonamedit.com/doctor.cfm/2454.html; Dorland's illustrated medical dictionary, 31st ed. Philadelphia: Saunders Elsevier; 2007.

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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-

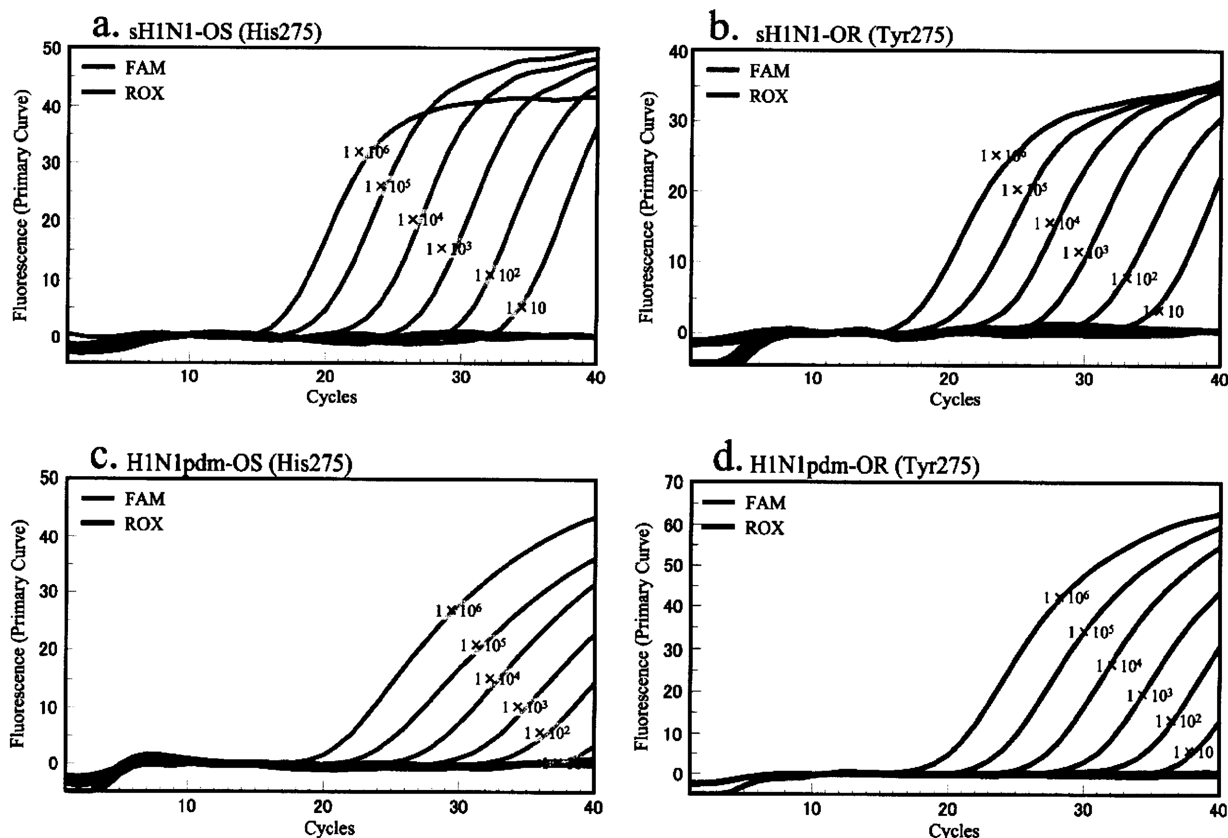

FIG. 1. Limit of detection of cycling probe real-time PCR with control plasmids. FAM fluorescence signals correspond to the oseltamivir-sensitive genotype (His275), and ROX fluorescence signals indicate the oseltamivir-resistant genotype (Tyr275). Control plasmids containing inserts of seasonal H1N1 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).

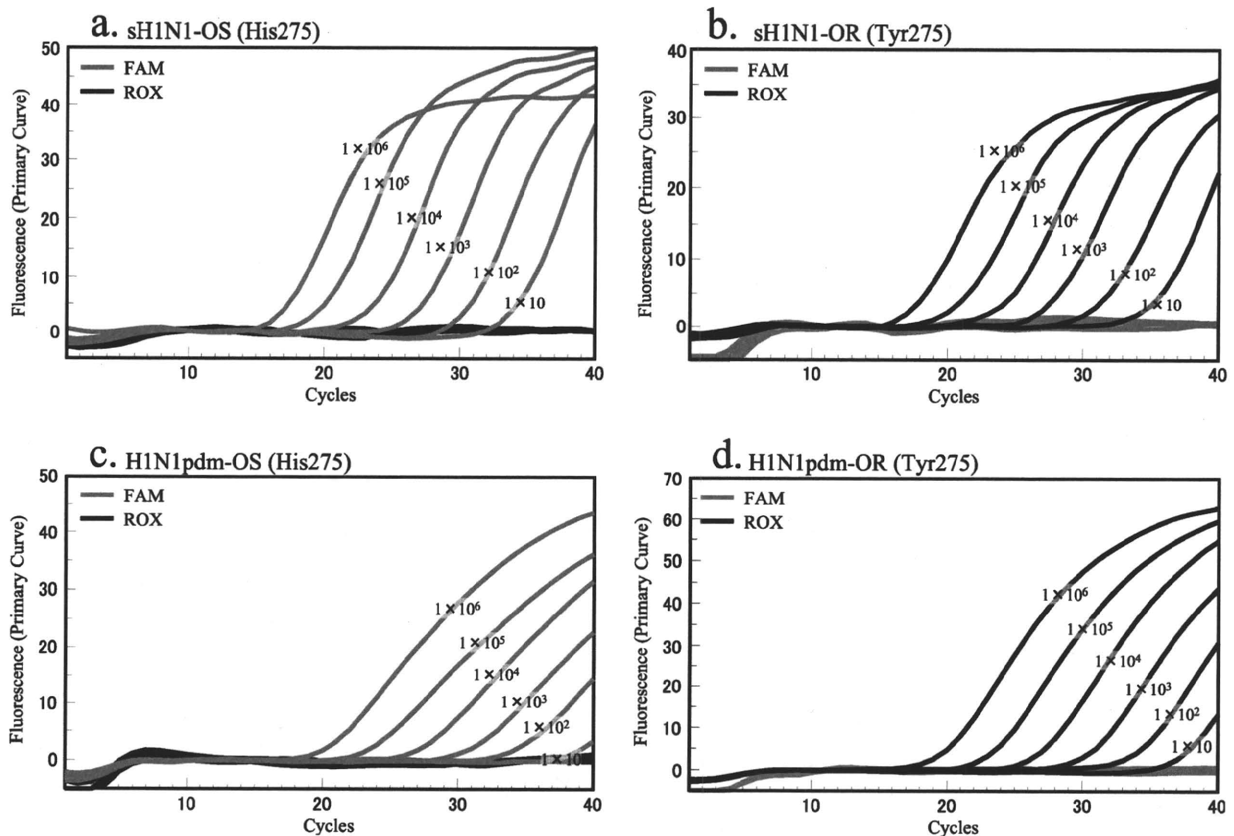

FIG. 1. Limit of detection of cycling probe real-time PCR with control plasmids. FAM fluorescence signals correspond to the oseltamivir-sensitive genotype (His275), and ROX fluorescence signals indicate the oseltamivir-resistant genotype (Tyr275). Control plasmids containing inserts of seasonal H1N1 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).

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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).

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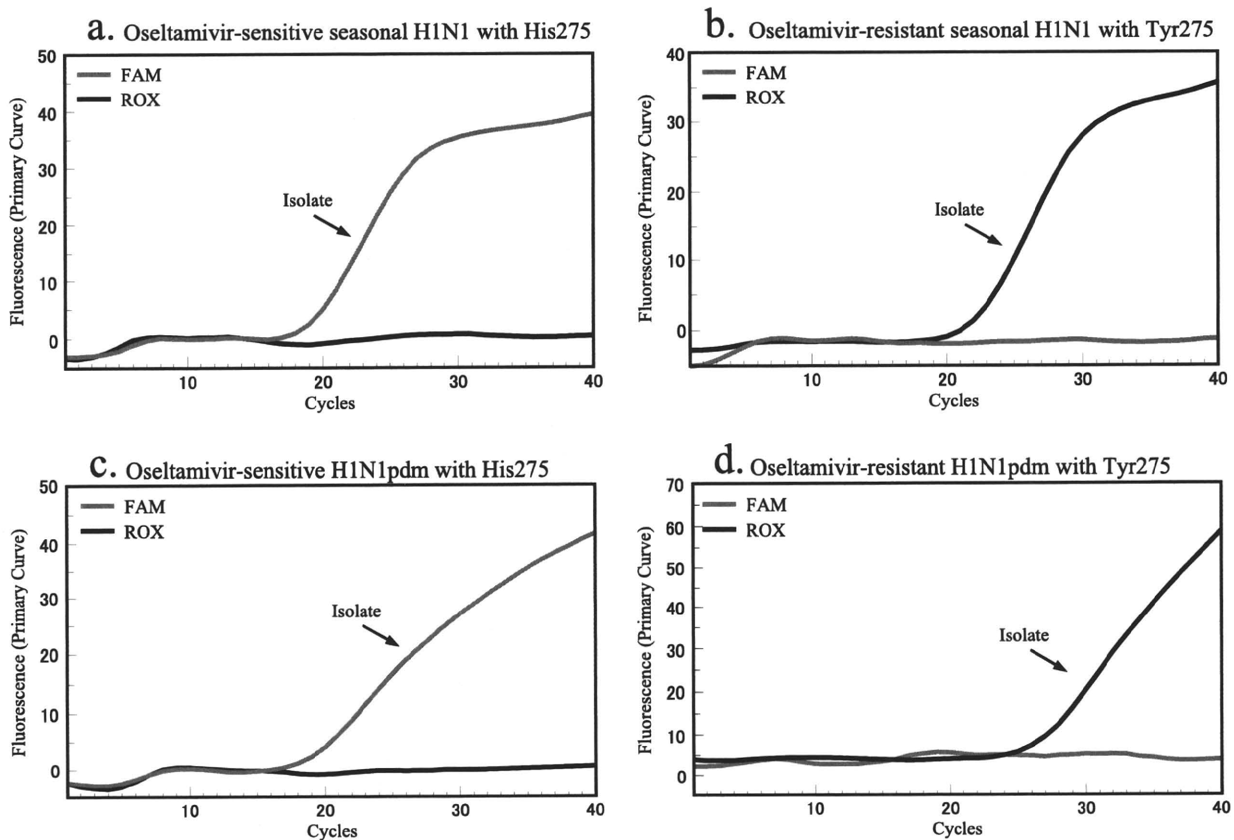

FIG. 2. Detection of oseltamivir-sensitive and -resistant isolates with H275 and H275Y in NA gene of influenza A (H1N1) virus. Oseltamivir-sensitive and -resistant viruses of seasonal H1N1 virus reacted with the FAM probe and the ROX probe, respectively (a and b). Oseltamivir-sensitive and -resistant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

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.

ACKNOWLEDGMENTS

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特集 新型インフルエンザ(パンデミックH1N1 2009)の教訓と今後の対策

齋藤　玲子

8．新型インフルエンザに対する抗インフルエンザ薬と耐性

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はじめに

本邦では，M２阻害薬とノイラミニダーゼ(NA)阻害薬がインフルエンザの予防・治療に使用されている．インフルエンザウィルスの薬剤耐性は，HIVや肝炎ウイルスと同様，作用蛋白の特定のアミノ酸が変異することにより生じる(実際にはRNAの１塩基置換)．しかし，薬剤によりRNAの突然変異が生じるのではなく，もともと変異をもつウイルスが一定の割合で存在し，投薬により変異ウイルスが選択されると考えられている[1)]．しかし，耐性ウイルスは，イオンチャネル機能の阻害(M２阻害薬)や酵素活性の低下(NA阻害薬)など，感受性株と比較すると何らかの不利益を生じるため，薬剤投与後に一過性に出現することはあっても(数～30%)，薬剤耐性株が市中で大流行することはないといわれてきた．しかしここ数年，それを覆すような出来事が起きている．アマンタジンやオセルタミビルに耐性の季節性インフルエンザが世界的に流行し，市中株のほぼ100%を占めるに至った．さらに，新型インフルエンザウィルス(以下，H1N1pdm)は発生当初からアマンタジン耐性である．これらのウイルスは，薬剤の選択圧には依存せずに，感受性株と同等のヒト-ヒト感染を起こす能力をもつウイルスである．

新型インフルエンザのM２阻害薬耐性(アマンタジン)

アマンタジン(シンメトレル®)は，A型インフルエンザの治療・予防薬であり，M２イオンチャネルを特異的にブロックする．M２チャネルはウイルス膜に存在する水素イオンチャネルであり，ウイルスの脱核と成熟の際にウイルス内部のpHを下げる．アマンタジンは，M２チャネルの内部をブロックするという説と，膜通過後のウイルス内面側をブロックするという２つの説がある[2)]．

アマンタジンへの耐性化は，M２チャネル内面の26，27，30，31位のアミノ酸のうちのどれか１つが変異することにより生じる．この変異により，水素イオンチャネル機能が低下し，増殖・伝播力が落ちると考えられる[2)]．このため，内服後の薬剤耐性出現率は数十%程度であり，人から人への感染は家族や施設内など限局的な範囲のみで，市中株中のアマンタジン耐性頻度は0.6～数%程度と低かった[3)]．しかし，2005～2006年以降，季節性A/H3N2(A香港型)とA/H1N1(ソ連型)インフルエンザにおいて，31位アミノ酸がセリンからアスパラギンに変異(S31N)したウイルスの大流行が起こり，市中株の60～100%がアマンタジン耐性となった[4,5)]．われわれは，この大流行したアマンタジン耐性株のHA遺伝子に，特有のアミノ酸変化が同時に起こっていることを報告した[4,5)]．さらには，遺伝子組み替えによる変異がほかの遺伝子にも起こり，これらがS31N変異株の増殖能低下を防いでいると考えられた[6)]．

新型インフルエンザH1N1pdmは，パンデミック発生当初からすべてアマンタジン耐性であることが知られ，治療にはアマンタジンを用いないよう米国では勧

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表1 2009～2010年シーズンに本邦各地で採取された新型インフルエンザH1N1pdmの薬剤耐性頻度(当教室調査)

県	H1N1pdm 陽性件数	S31Nアマンタジン耐性件数	%	H274Yオセルタミビル耐性件数	%
福島	53	53	100.0	0	0.0
新潟	75	75	100.0	0	0.0
群馬	28	28	100.0	0	0.0
京都	301	300	99.7	0	0.0
兵庫	62	62	100.0	0	0.0
長崎	103	102	99.0	0	0.0
合計	622	620/622	99.7	0/622	0.0

告された．われわれが日本各地で2009年に採取したH1N1pdmは，ほぼすべて(99.7%)がアマンタジン耐性であった(表1)．米国の調査においても，H1N1pdmのアマンタジン感受性株はごくわずかしか検出されていない[7]．

アマンタジン耐性であるH1N1pdmのM遺伝子は，ヨーロッパ系のブタインフルエンザ由来である[8]．1988～1989年頃に，すべてのヨーロッパ系A型ブタインフルエンザウイルスは，突然，S31N変異のアマンタジン耐性となった[9]．同時にM遺伝子の77位にも変異が起きていたが(R77Q)，この変異がチャネルの機能低下を是正しているのか定かではない．2000年以降もヨーロッパ系のブタインフルエンザウイルスでアマンタジン耐性は続き，今回，このM遺伝子がNA遺伝子とともに遺伝子組み換えで新型インフルエンザに入ってきた．ヨーロッパではブタにアマンタジンを投与した形跡はなく，なぜ突然この時期にアマンタジン耐性になったのか理由は判然としない．しかし，1930年代のH1N1インフルエンザ(WSNやPR 8株)は，アマンタジンが存在しなかったにもかかわらず，アマンタジン耐性であったことから，この変異は，遺伝子の組み合わせによってはウイルス増殖にそれほど不利とはならず，感受性株を凌駕する増殖能をもつと考えられる[10]．現在まで，どの遺伝子がS31N変異をサポートして耐性を維持しているのかは判明していない．今後の調査結果が待たれる．

NA阻害薬耐性(オセルタミビル，ザナミビル，ペラミビル，ラニナミビル)

NA阻害薬は，インフルエンザNA蛋白の宿主シアル酸に対する作用を競合的に阻害する．耐性化は，NA蛋白の特定の1アミノ酸変異による．本邦では，2001年にオセルタミビル(タミフル®)とザナミビル(リレンザ®)が認可され，インフルエンザ治療の主流となった．2010年にはペラミビル(ラピアクタ®)が発売され，ラニナミビル(CS-8958)も発売が近い．先に発売されたオセルタミビルとザナミビルを比べると，オセルタミビルで耐性が出現しやすいといわれる．ザナミビルは天然のシアル酸の形状に近い親水性のグリセロールをC 6位にもつが，オセルタミビルは，疎水性基を同部位にもつ．そのため，NA蛋白にオセルタミビルがドッキングするには追加的に活性部位の構造変化が必要となり，わずかなアミノ酸変異にも影響されやすくなるためといわれる[11]．

オセルタミビル耐性は，インフルエンザの型・亜型により耐性となるアミノ酸変異部位が異なり，臨床的にはA型N 1(H1N1pdmを含むH1N1，H5N1)ではH274Y変異，A型N 2(H3N2)ではR292K変異，E119V変異，N294S変異，B型ではR152K，D198N変異が知られてきた[12]．さらに，NA阻害薬の耐性は活性中心(active site)の変異か(292位)，枠組み蛋白(frame work)の変異(119位，198位，274位，294位など)かにより耐性の度合いが異なる．なお，本稿では，N 1とN 2のアミノ酸部位を合わせるため，N 2ナンバリングで表記する．活性中心の変異は高度耐性(約1万倍)となるが，酵素機能が著しく阻害されるので，伝播はしにくい．しかし，枠組み蛋白の変異は，低～中等度耐性(数倍～数百倍)となり，酵素活性はそれほど落ちないので，ある程度の伝播力をもつが流行はしないといわれてきた．しかし，2007～2008年には，ヨーロッパに端を発した274位変異(H274Y)のオセルタミビル耐性季節性H1N1株が流行を起こし，日本においてもその次のシーズンに大流行した[13]．この変異株はザナミビル

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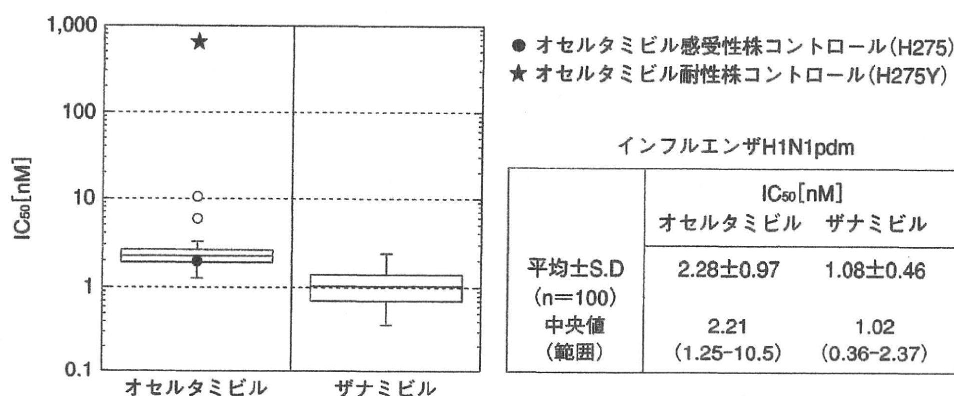

	IC50[nM]	
	オセルタミビル	ザナミビル
平均±S.D (n=100)	2.28±0.97	1.08±0.46
中央値 (範囲)	2.21 (1.25-10.5)	1.02 (0.36-2.37)

図1　H1N1pdmのノイラミニダーゼ阻害薬薬剤耐性試験
初診採取時のH1N1pdm株のIC50値(当教室調査)

には感受性である．大流行する薬剤耐性インフルエンザの特徴は，HAあるいは，NA遺伝子，そのほかの内部遺伝子に特徴的な変異をもつということである[14]．H274Y変異により，通常はNAの酵素機能が低下してしまうが，それを相殺する変異がウイルスゲノムのどこかに起こっていると考えられる．われわれは，HA遺伝子の193位に共変異が起こったことを報告したが[13]，米国の研究者は，オセルタミビル耐性株大流行の1～2シーズン前からNA蛋白にR222Q，V234Mという変異がみられており，これらが，NAの機能低下を相殺していると報告した[15]．一方，われわれはこの季節性H1N1耐性インフルエンザが臨床的にも「耐性」であり，オセルタミビルを投与して治療した場合に，罹患小児の有熱期間が無治療児とほぼ同等となることを報告した[16]．

今回のH1N1pdmは，オセルタミビル，ザナミビル双方に感受性である．われわれが行ったH1N1pdmに対する感受性検査では，それぞれ50%阻止濃度(IC50)がオセルタミビルに対して2.28 nM，ザナミビルに対して1.08 nMと感受性であることを示した(**図1**)．遺伝子レベルでも，初診時に採取された株からNAの274位変異は見つからなかった(**表1**)．

一方で，世界保健機関(WHO)の調査によるとH274Y変異オセルタミビル耐性株が全世界で約300件報告され(http://www.who.int/csr/disease/swineflu/updates/en/index.html)，日本では国立感染症研究所により2010年8月まで71件(1.1%)が報告された(http://idsc.nih.go.jp/iasr/influ.html)．この変異株は，季節性H1N1と同様に，ザナミビルに対しては感受性であるが，オセルタミビルに対しては500～700倍の感受性の低下がある[17]．主にオセルタミビルの治療内服後(特に免疫不全者の長期投与)や予防投与者で検出されており，人から人への感染は限定的である．これまでの知見では，H1N1pdmのH274Y変異株は，感受性株に比べNA酵素活性が落ちており，*in vitro*の増殖効率が悪い[18]．インフルエンザの動物モデルであるフェレットの実験では，同じケージに動物を入れて直接の接触があった場合にはH274Y耐性株による感染伝播が起こったが，金網で仕切られ飛沫感染を擬した状態ではインフルエンザの感染はなかった．対照実験の感受性株では，飛沫感染によりフェレットでインフルエンザを発症している．このため，H1N1pdmのH274Y耐性株の伝播力は，感受性株に比べて劣っていると考えられる．結果的には，H274Y耐性株によるH1N1pdmの大流行の兆しは未だないといえ，酵素活性を上昇させる次なる遺伝子変異を待たねばならない．

2010年に本邦で発売，または発売予定のペラミビルとラニナミビル(本邦CS-8958，米国R-125489)は，両薬剤ともH1N1pdmには感受性である(**表2**)[17]．しかし，H274Y変異を有するH1N1pdmに対しては，ペラミビルの感受性は10倍程度低下している[17]．ラニナミビルには感受性の低下はない．今後，ペラミビルに対するこの10倍程度の感受性の低下が臨床的に影響するのか否か検証が必要である．

おわりに

IC50でスクリーニングを行い，シークエンスでNA遺伝子の変異部位を確かめるという手順で，様々な変異が見つかってきている．さらに，H1N1pdmの大流行によりNA阻害薬の使用量が全世界的に増えているためか，V116，I117，Q136，D198，I222，N294位など，

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表2　季節性H1N1と新型H1N1pdmのペラミビル，ラニナミビル(R-125489)，A-315675に対する感受性—発光法，蛍光法，比色法によるIC_{50}値の比較

Virus strain	Subtype	NA change	Peramivir			R-125489			A-315675		
			CL[b]	FL[c]	CM[a]	CL	FL	CM	CL	FL	CM
			Mean±SD[e] (Fold)	Mean±SD (Fold)	Mean±SD (Fold)	Mean±SD (Fold)	Mean±SD (Fold)	Mean±SD (Fold)	Mean±SD (Fold)	Mean±SD (Fold)	Mean±SD (Fold)
A/Washington/10/2008[f]	H1N1	WT[g]	0.11±0.01	0.18±0.07	0.75±0.23	0.14±0.01	0.53±0.08	0.79±0.03	0.16±0.01	0.51±0.15	1.03±0.20
A/North Carolina/02/2009	H1N1	WT	0.08±0.01	0.19±0.05	0.70±0.05	0.17±0.01	0.61±0.05	0.86±0.10	0.18±0.01	0.51±0.05	0.75±0.50
			(1)	(1)	(1)	(1)	(1)	(1)	(1)	(1)	(1)
A/New York/18/2009	H1N1pdm[h]	WT	0.12±0.04	0.32±0.06	0.41±0.03	0.49±0.05	0.96±0.13	0.74±0.25	0.43±0.05	0.74±0.14	0.90±0.32
			(1)	(1)	(1)	(1)	(1)	(1)	(1)	(1)	(1)
A/W ashington/29/2009	H1N1pdm	WT	0.14±0.04	0.20±0.04	0.49±0.10	0.49±0.06	0.74±0.09	1.57±0.13	0.35±0.05	0.53±0.04	1.31±0.030
A/Singapore/91/2009	H1N1pdm	WT	0.11±0.02	0.14±0.07	0.55±0.09	0.30±0.04	0.51±0.01	0.90±0.33	0.27±0.01	0.46±0.04	1.40±0.18
			(1)	(1)	(1)	(1)	(1)	(1)	(1)	(1)	(1)
A/North Carolina/01/2009	H1N1	H275Y	13.75±0.88	149.59±9.40	105.02±8.50	0.34±0.04	0.92±0.08	0.89±0.12	0.84±0.12	1.93±0.19	2.32±0.34
			(125)	(831)	(140)	(2)	(2)	(1)	(5)	(4)	(2)
A/Montana/02/2009	H1N1	H275Y	14.96±0.52	197.10±24.72	83.40±1.34	0.34±0.02	1.05±0.01	0.82±0.11	1.00±0.34	2.60±0.08	1.89±0.31
			(1.36)	(1.095)	(111)	(2)	(2)	(1)	(6)	(5)	(2)
A/Osaka/180/2009	H1N1pdm	H275Y	13.06±2.60	97.56±13.40	194.96±10.88	0.48±0.05	1.43±0.06	3.32±0.42	0.91±0.05	2.44±0.31	4.08±0.56
			(93)	(488)	(398)	(1)	(2)	(2)	(3)	(5)	(3)
A/Washington/29/2009	H1N1pdm	H275Y	12.50±1.92	150.24±4.00	162.58±8.06	0.82±0.06	1.19±0.04	2.14±0.44	1.62±0.05	1.76±0.21	2.07±0.28
			(89)	(751)	(332)	(2)	(2)	(1)	(5)	(3)	(2)
A/Hong Kong/2369/2009	H1N1pdm	H275Y	9.24±1.13	128.15±1.97	161.79±38.09	0.78±0.02	1.08±0.06	2.16±0.57	1.09±0.09	1.67±0.06	3.56±0.48
			(66)	(641)	(330)	(2)	(1)	(1)	(3)	(3)	(3)
A/Singapore/57/2009	H1N1pdm	H275Y	11.79±0.41	121.76±2.91	84.80±14.39	0.74s±0.03	0.86±0.02	1.12±0.11	1.06±0.08	1.39±0.02	1.58±0.08
			(84)	(609)	(173)	(2)	(1)	(1)	(3)	(3)	(1)

a：As measured by mean IC_{50}(nM) ± standard deviation (SD) and fold change compared to the conesponding reference wild type drug sensitive virus.
b：CL, chemiluminescent assay.
c：FL, fluorescent assay.
d：CM, colorimetric assay.
e：The IC50 values were calculated from at teast therec independent experiments for the CL and FL assays and at least two independent experiments for the CM assay.
f：Bold, wild type reference drug sensitive virus.
g：WT, wild type.
h：pdm, pandemic.
なお，A-315675は日本で発売未定．

(文献17より引用)

これまであまりみられなかった変異も見つかっており，遺伝子変異スクリーニングの幅を広げる必要がでている[7]．

H1N1pdmは元々アマンタジン耐性であるため，NA阻害薬耐性が加われば，2剤耐性である．さらには，NA阻害薬の中でも化学構造式の相似により変位の部位によっては相互耐性になる場合がある．耐性に関するスクリーニングは今後さらに多様化し，スピードが求められるようになる．インフルエンザはHIVやB型肝炎とは異なり，病期が1週間程度の急性疾患であるため，多剤耐性インフルエンザが患者の予後に大きく影響することは少ないと考えられるが，今後，重症者や免疫不全患者において多剤併用療法ストラテジーを考える必要が生じるかもしれない．

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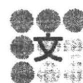

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ORIGINAL ARTICLE

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

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

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

Methods

Study procedures

Family doctors, pediatricians, and physicians at 15 clinics that belong to the Influenza Study Group of the Japan Physicians Association participated in the study. Patients were enrolled from December 7, 2007 through March 27, 2008 in the 2007–2008 season, from December 6, 2008 through February 14, 2009 in the 2008–2009 season, and from August 11, 2009 through January 31, 2010 in the 2009–2010 season. Patients who reported to any of our 15 clinics with an influenza-like illness manifesting any two of the following symptoms—body temperature ≥37.5°C, rhinorrhea, sore throat, cough, general fatigue, loss of appetite, or headache—were tested by a commercial antigen detection kit. From all outpatients with influenza, diagnosed by antigen detection kit and/or clinical symptoms and without severe underlying diseases such as chronic obstructive pulmonary disease or chronic heart disease, those who received oseltamivir or zanamivir within 48 h after the onset of symptoms were registered in this study after providing informed consent. Excluded from the analysis were four patients in serious condition who were sent immediately to a hospital.

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

Specimens from throat swabs, nasal swabs, nasal aspirates, or blown nasal discharge were subjected to antigen detection and virus isolation. Of the commercially available antigen detection kits based on immunochromatography, Capilia FluA+B (Alfresa Pharma), QuickVue Rapid-SP influ (DS Pharma Biomedical), QuickNavi-Flu (Denka Seiken), and Imuno Ace Flu (Touns), were mainly used.

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

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

Oseltamivir (75 mg for adults and for children who weighed >37.5 kg and 2 mg/kg for children who weighed <37.5 kg) was taken orally twice per day for 5 days. Zanamivir (10 mg for adults and for children aged 5 years or over) was inhaled twice per day for 5 days. Antipyretics were not administered, but acetaminophen was used temporarily in the case of emergency.

Age, sex, vaccination status, results of the antigen detection test kit, and body temperature were recorded for all patients. The date and time of the onset of fever, the date and time of administration of oseltamivir or zanamivir, and the resolution of fever were recorded by the physician, patient, or an attending family member. The first time that a patient reported a fever (temperature 37.5°C) was defined as the time of onset. Patients were asked to measure body temperature at least three times per day (8:00 a.m., 2:00 p.m., and 8:00 p.m.). The time at which a body temperature <37.5°C was attained and maintained for more than 24 h was defined as the time when the patient became afebrile. The highest body temperature during the course of the disease was also recorded. For clinical symptoms other than fever, the presence or absence of the following symptoms were noted by the doctor when influenza was diagnosed: cough, fatigue, rhinorrhea, sore throat, myalgia, headache, loss of appetite, vomiting, and diarrhea.

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

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

[a] Vaccination for seasonal influenza

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

Statistical analysis

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

Results

Patient characteristics

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

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

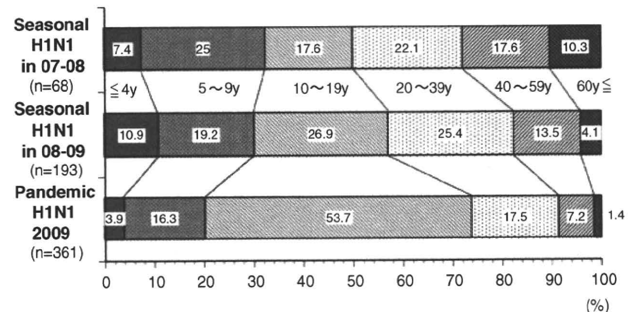

Fig. 1 Age distribution of patients with seasonal H1N1 in the 2007–2008, 2008–2009, and pandemic H1N1 2009 seasons. The percentage of patients aged 10–19 years was significantly higher for pandemic H1N1 than for seasonal H1N1 in the 2007–2008 and 2008–2009 seasons

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

Clinical symptoms

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

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

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

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

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

Table 3 Patient characteristics by treatment

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

[a] Vaccination for seasonal influenza

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

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

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

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