Selection pressure and evolutionary rate analyses

Selection analysis was carried out using probabilistic models of codon substitution implemented in the CodeML program included in the PAML package, version 4.0 [44]. This program employs likelihood models that account for heterogeneous substitution rate ratios (ω = non-synonymous/synonymous substitution or d*N*/d*S*) among sites. In this study, we implemented models M0, M1a, M2a, M3, M7, and M8, which were previously tested for their robustness in testing for positive selection [45, 46]. An $\omega > 1$ is considered an indication of positive selection, whereas an $\omega < 1$ implies absence of positively selected sites. Three comparisons were conducted. The first was the neutral model (M1a), which assumes a proportion p_0 of conserved sites with $0 < \omega_0 < 1$ and a proportion $p_1 = 1-p_0$ of neutral sites with $\omega_1 = 1$, against the selection model (M2a), allowing for a third class of sites with $\omega_2 > 1$ (positively selected sites) and a proportion $p_2 = 1-p_0-p_1$. Model M0, assuming a single ω for all sites was compared with M3, which uses an unconstrained discrete distribution with three site classes (ω_0, ω_1, ω_2 with proportions p_0, p_1, p_2, respectively). The third test compared M7 (β model), which uses β distribution with parameters p and q to account for variable ω_0 in the interval of zero to unity against M8, which adds to the β model an extra class of sites with $\omega > 1$ (with p_2 and ω_2). The Bayes approach was then used to calculate the posterior probability that each site identified to be under positive selection ($\omega > 1$) belonged to this class of sites [45]. The likelihood ratio test (LRT) was used to determine whether models (M2a, M3, and M8) allowing for positive selection sites ($\omega > 1$) provide a significantly better fit to the data than the alternative models that do not allow for positive selection or null models (M0, M2a, and M7). When two models are nested, the LRT compares twice the likelihood difference ($2\Delta l$) with a X^2 distribution with the degrees of freedom (df) equal to the difference in the number of free parameters between the two models (check ref. [40, 42, 43] for detailed explanation of the models and their parameters). LRT was performed using the PAML program [44, 45]. Estimates of evolutionary rate (nucleotide substitution rate) were obtained by the maximum-likelihood method using a single dated tips model that assumes a constant rate of substitution [26]. This model was implemented in the BASEML program included in the PAML package, version 4.0 [44].

N-Glycosylation analysis

Potential *N*-glycosylation sites (amino acids Asn-X-Ser/Thr, where X is not Pro) were predicted using the NetN-Glyc server 1.0 (available on the www.cbs.dtu.dk website).

Nucleotide accession numbers

The nucleotide sequences used in this study are available in the GenBank database under accession numbers AB271703, AB271704, and AB438225 to AB438363.

Results

Influenza virus A subtypes and amantadine-resistance rates

A total of 1,609 influenza virus isolates were obtained during the eight influenza seasons from 2000 to 2007 (Table 1). Of these, influenza A accounted for 65% ($n = 1041$) and 35% ($n = 568$) were influenza B. Of the influenza A virus isolates, 766 (75.4%) were H3N2 and 275 (24.6%) were H1N1. H3N2 viruses were detected in all seasons, while H1N1 viruses were isolated during five of the eight seasons (no H1N1 viruses were detected in the 2002/2003, 2003/2004, and 2004/2005 seasons). The average age among patients from whom the virus could be isolated was 6.6 ± 4.5 years old, compared to 5.2 ± 4.6 years old for patients for whom no virus was isolated (data obtained from 2001/2002 to 2006/2007 seasons). The vaccination rate among the patients included in the study was 5, 22, 39, 40, 48, and 49%, for the 2001/2002, 2002/2003, 2003/2004, 2004/2005, 2005/2006, and 2006/2007 seasons, respectively.

Amantadine-resistance rates among H3N2 viruses were between 0.7 and 3.4% during 1999/2000–2004/2005 and surged to 100% in 2005/2006 and 79.4% in 2006/2007. In the case of H1N1, amantadine-resistant viruses were not detected until the 2006/2007 season, when 48.2% of the isolates were found to be resistant (Table 1). The M2 gene sequence analysis revealed that all amantadine-resistant H3N2 and H1N1 viruses had a single amino acid change from Ser to Asn at residue 31 (S31 N) in the M2 protein.

Sequence and phylogenetic analysis of the HA1 subunit of the HA gene

A total of 88 H3N2 HA1 sequences (including amantadine-sensitive and resistant viruses), and 56 H1N1 HA1 sequences (including amantadine-sensitive and resistant viruses) in addition to reference sequences of vaccine strains for each subtype were employed in the phylogenetic analysis.

For H3N2, the phylogenetic tree was highly branched, but evolved sequentially in a single linear trunk (Fig. 1a). The tree was interrupted only once in the 2001/2002 season with abortion of the A/Panama/2007/1999 lineage, and the sequences of the 2002/2003 season evolved from those of

Table 1 Incidence of influenza A subtypes and amantadine-resistant isolates during the study period

Season	H3N2		H1N1	
	No. of isolates	Amantadine-resistance (%)	No. of isolates	Amantadine-resistance (%)
1999–2000	59	2 (3.4)	90	0 (0)
2000–2001	12	0 (0)	15	0 (0)
2001–2002	44	1 (2.3)	59	0 (0)
2002–2003	178	2 (1.1)	0	0
2003–2004	167	2 (1.2)	0	0
2004–2005	142	1 (0.7)	0	0
2005–2006	101	101 (100)	55	0 (0)
2006–2007	63	50 (79.4)	56	27 (48.2)
Total	766	159	275	27

2000/2001, characterized by retaining the signature amino acid substitutions found in the 2000/2001 season strains (e.g. I144N) and loss of those of the 2001/2002 season strains (A106V, N144D, S186V), with the appearance of A/Fujian/411/2002-like strains (Fig. 1a). In the 2006/2007 season, the trunk split into two branches: one contained amantadine-sensitive viruses, and the other accommodated the resistant ones. In total, amino acid mutations at 62 sites in the HA1 subunit were detected for the whole study period (Fig. 1b). Of these, 21 amino acid changes were retained for two or more successive years (Fig. 1c), and 14 of them belonged to the receptor-binding domain (RBD; 190 helix: residues 192 and 193; 220 loop: residues 222, 225, 226, and 227) and/or one of the reported antigenic sites (antigenic site A: residues 131, 144, and 145; B: residues 155, 156, 189, 192, and 193; D: residue 202; and E: residue 83) in the globular head [7, 39, 41].

In the case of H1N1, a multi-furcated tree was formed (Fig. 2a). The tree in the A/New Caledonia/20/1999-like lineage was interrupted in the 2001/2002 season, after which no H1N1 viruses were detected for three consecutive seasons, and the tree evolved again in the 2005/2006 season from the sequences of the 1999/2000 season. However, the trunk was again interrupted and eventually evolved into three major branches in the 2005/2006 and the 2006/2007 seasons. One of the branches included A/Solomon Islands/3/2006, which had quit circulating during the 2006/2007 season, and one of the two branches in the 2006/2007 season (harboring amantadine-sensitive viruses) contained A/Brisbane/59/2007, the recommended vaccine strain for the southern hemisphere in 2008. In total, amino acid changes at 36 sites in the HA1 subunit of H1N1 were detected over the study period (Fig. 2b). Only 11 of these were retained for $\geq$2 consecutive years (Fig. 2c), of which four amino acid changes occurred in the RBD (190 helix: residue 190) or one of the antigenic sites (site Sa: residue 124; and site Ca1: residues 169 and 270) located in the globular head [7, 41, 47].

Positive selection and evolutionary rate analyses

At the amino acid level, the average dN/dS (avg. ω) for the HA1 subunit of H3N2 viruses ranged from 0.41 to 0.44 in all codon substitution models (Table 2). Thus, a non-synonymous substitution has about 41–44% as much chance as synonymous mutations of being fixed. On the other hand, for the H1N1 viruses, the average dN/dS ratio (avg. ω) ranged between 0.25 and 0.29, implying that a non-synonymous substitution has about 25–29% as much chance as a synonymous mutation of becoming fixed (Table 2). For HA1 of both H3N2 and H1N1, the selection M2a and M8 models successfully detected positively selected sites ($\omega_2 = 1.48$ with $p_2 = 0.22$, and $\omega_2 = 1.52$ with $p_2 = 0.2$, respectively, for H3N2, and $\omega_2 = 7.16$ with $p_2 = 0.005$, and $\omega_2 = 7.1$ with $p_2 = 0.005$, respectively, for H1N1), but did not significantly provide a better fit to the data when compared with the alternative neutral M1a and beta distribution M7 models, respectively, as determined by the LRT (Table 2). On the other hand, the discrete model (M3) provided a significantly better fit to the data in comparison with the one ratio model (M0); the LRT test statistic for this comparison was $2\Delta l = 2 \times [-2273.09 - (-2285.04)] = 23.9$, with P value < 0.001 with $df = 2$, for H3N2, and $2\Delta l = 27$, with P value < 0.001 with $df = 2$, for H1N1 (Table 2).

In the case of H3N2, the M3 model suggested that approximately 20% ($p_2 = 0.2$) of the sites were under positive selection ($\omega_2 = 1.5$). At a threshold of 95%, only five amino acid sites, namely residues: 50, 131 (antigenic site A), 144 (antigenic site A), 145 (antigenic site A), and 220 (antigenic site B), were under positive selection. In the case of H1N1, the M3 model estimated that $\sim$15% ($p_1 = 0.15$) of the sites were under weak diversifying selection, with $\omega_1 = 1.13$, and only $\sim$0.005% ($p_2 = 0.0049$) of sites were under high positive selection, with $\omega_2 = 7.26$. At 95% threshold, residues: 144, 149, 163 (antigenic site Sa), and 190 (RBD) were positively selected.

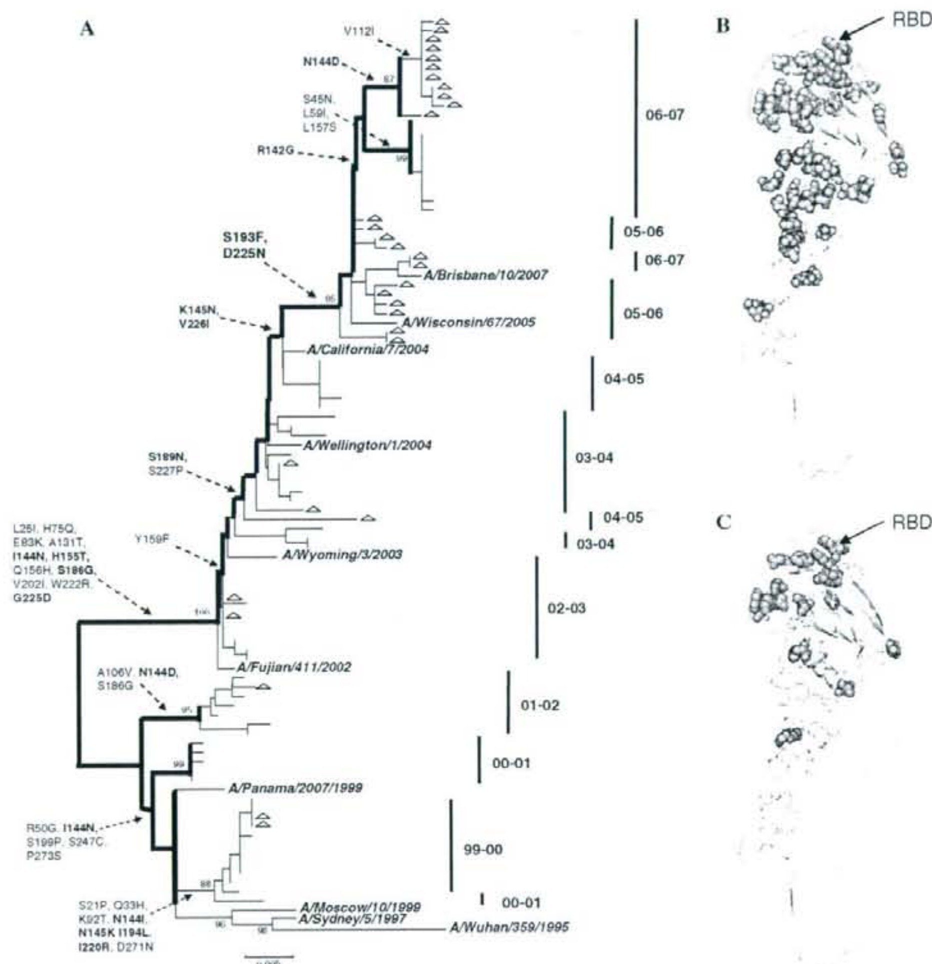

Fig. 1 Sequence analysis of the HA1 subunit of the hemagglutinin (HA) glycoprotein of the human influenza A/H3N2 viruses isolated in Niigata, Japan, during 1999–2007. **a** Phylogenetic tree analysis of the HA1 fragment from strains isolated in Niigata, rooted at A/Wuhan/359/1995. Reference vaccine strains are denoted in italics. A *delta* sign indicates amantadine-resistant strains. Characteristic amino acid mutations are shown on the trunk of the tree (mutations occurring within antigenic sites or RBD are indicated in *bold*). Bootstrap values greater than 70% are also shown. **b** Structure of the HA1 subunit of human H3 (PDB: 2hmg; A/Aichi/1968). The surface-filling models represent all sites at which amino acid substitutions were observed for the whole study period among Niigata isolates. The *grey shading* indicates the RBD. **c** The structure of the HA1 subunit showing sites at which mutations were retained for two or more years. All protein structure figures were generated using the Pymol program [9]

The rate of substitution in the HA1 subunit was 4.58×10^{-3} nucleotide substitutions/site per year (95% confidence interval [CI], 3.9×10^{-3}–5.26×10^{-3}) for H3N2 and 3.83×10^{-3} nucleotide substitutions/site per year (95% CI 3.35×10^{-3}–4.3×10^{-3}) for H1N1 as estimated by the tip date analysis. Furthermore, the ratio of transitions to transversions (ts/tv) for H3N2 was almost half that of H1N1, 3.79–3.86 versus 6.96–7.35, respectively.

N-Glycosylation sites

Nine putative *N*-glycosylation sites (residues: 22, 38, 63, 122, 126, 133, 165, 246, and 285) were identified in the HA1 subunit of the H3N2 isolates (Table 3). These sites were found to be conserved among all isolates obtained in the eight seasons of the study. An additional site at position 144, due to an I144N substitution within antigenic site A

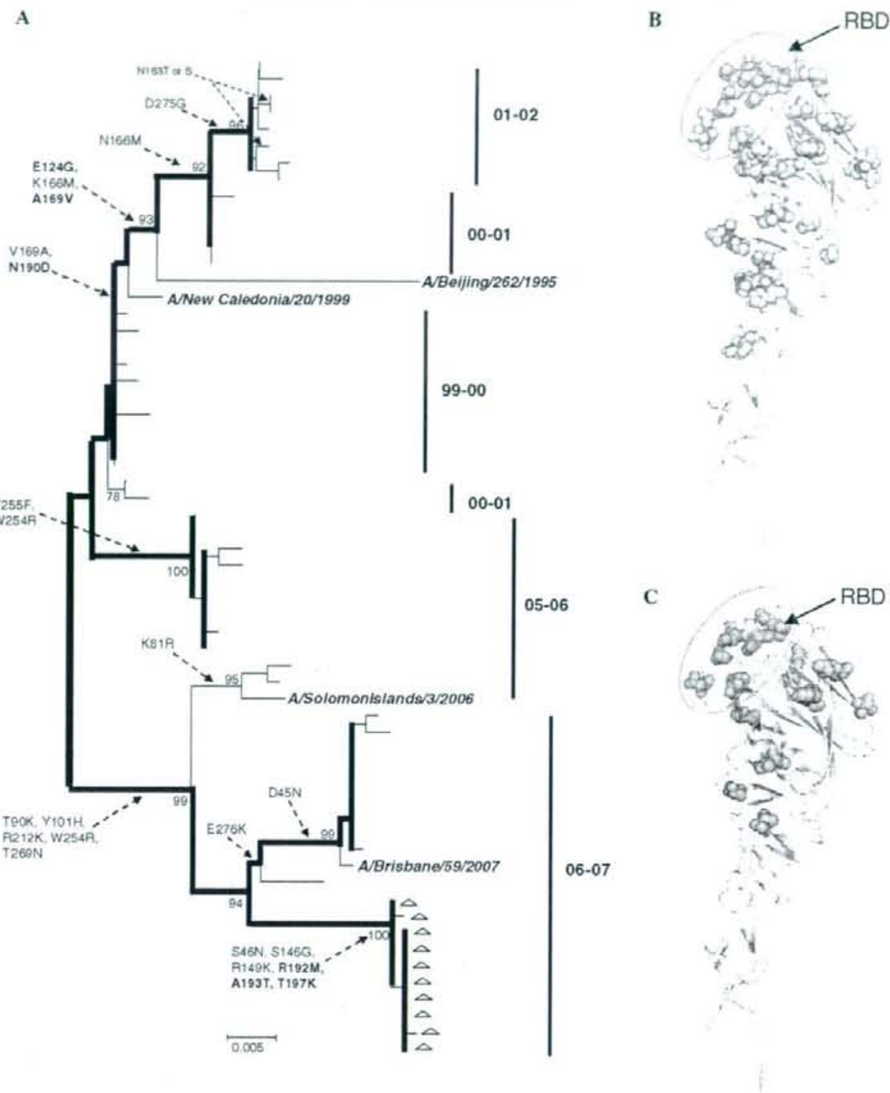

Fig. 2 Sequence analysis of the HA1 subunit of the hemagglutinin (HA) glycoprotein of the human influenza A/H1N1 viruses isolated in Niigata, Japan, during 1999–2007. **a** Phylogenetic tree analysis of the HA1 fragment from strains isolated in Niigata, rooted at A/Beijing/262/1995. Reference vaccine strains are denoted in italics. A *delta* sign indicates amantadine-resistant strains. Characteristic amino acid mutations are shown on the trunk of the tree (mutations occurring within antigenic sites or RBD are indicated in *bold*). Bootstrap values greater than 70% are also shown. **b** Structure of the HA1 subunit of human H1 (PDB: 1ruz; human 1918 HA expressed using a synthetically made gene). The surface-filling models represent all sites at which amino acid substitutions were observed for the whole study period among Niigata isolates. The *grey shading* indicates the RBD. **c** The structure of the HA1 subunit showing sites at which mutations were retained for two or more years. All protein structure figures were generated using the Pymol program [9]

[39], was acquired by the strains collected in the 2000/2001 season. This *N*-glycosylation site was temporarily lost in the 2001/2002 season, due to an N144D substitution, but was then retained in the following seasons until the 2006/2007 season, when only the amantadine-resistant viruses lost it again. Another *N*-glycosylation site (amino acid

Table 2 Log-likelihood values and parameter estimates for the selection analysis of the HA1 subunit of the HA genes of human influenza A

Subtype	Model	p [a]	l [b]	P value for LRT	Estimates of parameters [c]	Avg ω	ts/tv
H3N2	M0 (one ratio)	1	−2285.04	<0.0001	$\omega = 0.4327$	0.4327	3.85955
	M3 (discrete)	5	−2273.09		$p_0 = 0.27100, p_1 = 0.52312 (p_2 = 0.20588), \omega_0 = 0.01554, \omega_1 = 0.22964, \omega_2 = 1.52649$	0.4388	3.84203
	M1a (neutral)	2	−2273.60	0.6	$p_0 = 0.61684 (p_1 = 0.38316), \omega_0 = 0.04099 (\omega_1 = 1)$	0.4084	3.79393
	M2a (Positve selection)	4	−2273.09		$p_0 = 0.77498, p_1 = 0.0 (p_2 = 0.22502), \omega_0 = 0.13644 (\omega_1 = 1), \omega_2 = 1.48011$	0.4388	3.84204
	M7 (beta)	2	−2273.65	0.57	$p = 0.01570, q = 0.02025$	0.4176	3.81628
	M8 (beta and ω)	4	−2273.09		$p_0 = 0.79370, p = 1.47871, q = 7.83366 (p_2 = 0.20630)$ $\omega_2 = 1.52369$	0.4386	3.84305
H1N1	M0 (one ratio)	1	−1891.75	<0.0001	$\omega = 0.29098$	0.29098	7.10722
	M3 (discrete)	5	−1878.27		$p_0 = 0.84459, p_1 = 0.15045 (p_2 = 0.00496), \omega_0 = 0.09738, \omega_1 = 1.12535, \omega_2 = 7.25778$	0.2876	7.36490
	M1a (neutral)	2	−1880.85	0.07	$p_0 = 0.79775 (p_1 = 0.20225), \omega_0 = 0.06380 (\omega_1 = 1)$	0.2531	6.96159
	M2a (positive selection)	4	−1878.29		$p_0 = 0.81250, p_1 = 0.18240 (p_2 = 0.0051), \omega_0 = 0.08221 (\omega_1 = 1), \omega_2 = 7.16266$	0.2857	7.35049
	M7 (beta)	2	−1881.19	0.06	$p = 0.05925, q = 0.16792$	0.2608	7.01889
	M8 (beta and ω)	4	−1878.41		$p_0 = 0.99477, p = 0.13758, q = 0.41013 (p_2 = 0.00523)$ $\omega_2 = 7.09862$	0.2871	7.35652

LRT log likelihood ratio test for each comparison

[a] The number of free parameters in each model

[b] Log likelihood

[c] The parameters in parantheses are not free parameters

position 45) was exclusively found in amantadine-sensitive isolates in the 2006/2007 season. In total, four new predicted *N*-glycosylation sites (45, 122, 133, and 144) that were not present in A/Wuhan/359/1995, the vaccine strain for the 1996/1997 season, were found until the 2006/2007 season (Fig. 1).

In the case of H1N1, seven potential *N*-glycosylation sites (residues: 20, 21, 33, 63, 94, 130, and 163) were conserved throughout the study period except in the 2001/2002 season, when some of the circulating strains lost glycosylation at position 163 within antigenic site Sa (Table 3) [7].

Discussion

During the eight influenza epidemics from 2000 to 2007, H3N2 viruses were the dominant influenza A subtype in five of the eight seasons. The prevalence of amantadine-resistance among H3N2 viruses increased dramatically from 0.7 to 4.3% during the first six seasons to 100% in the 2005/2006 season, in association with dual mutations in the HA1 protein at residues 193 and 225 within the RBD (named clade N) [30, 32]. This high prevalence of resistant viruses was also found in other Asian countries, USA, and Canada [10, 22]. However, viruses sensitive to amantadine fell within the clade N in the HA1 phylogeny in the 2006/2007 season, suggesting a possibility of reverting back from resistance to sensitivity due to continuing viral evolution or competitive disadvantages of resistant viruses against sensitive ones. Amantadine-resistant H1N1 viruses also emerged in the 2006/2007 season, and their HA1 commonly possessed three amino acid substitutions (R192M, A193T, A197K) that were located in the 190 loop of the RBD [47] as in our previous report [32].

The surge in the prevalence of resistant viruses in Japan occurred despite the decrease in amantadine usage from 2.7 million treatment courses in the 2002/2003 season to only 0.1 million in the 2005/2006 season [31], supporting the notion that drug selection pressure was not the sole cause of this sharp rise in resistance rate, but some advantageous mutations located elsewhere in the viral genome might have contributed [33]. As a case in point, the increased prevalence of amantadine-resistant strains was associated with a common amino acid substitution at residue 193 within the RBD for both H3N2 and H1N1. An amino acid mutation at this position was also found in clade I A/H5N1 viruses, which were also resistant to amantadine [38]. This suggests a specific contribution by the substitution at this residue to the wide spread of resistance.

Phylogenetic analysis of the HA1 fragments for both H3N2 and H1N1 subtypes showed that the major cluster of

Table 3 *N*-Glycosylation sites predicted in the HA1 protein of influenza A isolates

Subtype	Season	Amino acid position[a]
H3N2	1999/2000	22,38,63, **122**, **126**, **133**,165,246, 285
	2000/2001	22,38,63, **122**, **126**, **133**, **144**,165,246, 285
	2001/2002	22,38,63, **122**, **126**, **133**,165,246, 285
	2002/2003	22,38,63, **122**, **126**, **133**, **144**,165,246, 285
	2003/2004	22,38,63, **122**, **126**, **133**, **144**,165,246, 285
	2004/2005	22,38,63, **122**, **126**, **133**, **144**,165,246, 285
	2005/2006	22,38,63, **122**, **126**, **133**, **144**,165,246, 285
	2006/2007	*S*[b]: 22,38,45,63, **122**, **126**, **133**, **144**,165,246, 285
		R[b]: 22,38,63, **122**, **126**, **133**, 165,246, 285
H1N1	1999/2000	20,21,33,63,94,130, **163**
	2000/2001	20,21,33,63,94,130, **163**
	2001/2002	20,21,33,63,94,130, **163**[c]
	2002/2003	NA[d]
	2003/2004	NA
	2004/2005	NA
	2005/2006	20,21,33,63,94,130, **163**
	2006/2007	20,21,33,63,94,130, **163**

[a] Bold numbers represent an antigenic binding site

[b] *S* indicates amantadine-sensitive strains and *R* denotes resistant ones

[c] some of the strains lost *N*-glycosylation at this position (3/8)

[d] NA, not applicable; no H1N1 viruses were isolated in these seasons

any given season did not harbor any sequences from the previous season. Moreover, during the study period, influenza viruses were detected only between December and May in Niigata, showing a seasonal pattern. These observations suggested that viruses circulating in each season in Niigata were derived from newly imported strains (external introduction) rather than continuing circulation of the endemic strains from the previous seasons. This seems to be a common feature of seasonal epidemics in temperate-climate areas [25, 27, 29]. On the other hand, the case is different in countries where influenza circulates all year round, such as in Southeast Asia. We previously showed that local epidemic strains in Vietnam from a certain season clustered phylogenetically with some strains from the previous epidemics, providing evidence for local persistence of influenza strains in tropical regions [22].

Amino acid changes in the HA1 subunit of both H3 and H1 were stochastic and scattered all over the protein, and approximately only one-third of these mutations were retained for two or more successive seasons. As most of the retained mutations were confined to the globular part of the HA1 domain, improvement of fitness and evasion of neutralization by the host antibodies were expected [20]. This supports the theory of positive Darwinian selection proposed for influenza A viruses [5, 12]. The total number of mutations in the HA1 of H3 was greater than that of H1, which suggests that a higher selective pressure is being imposed on H3 [43], and this explains the necessity for more frequent updates in the vaccine strains for the H3N2 subtype. Moreover, the characteristic linear trunk in the H3 tree in comparison with the multi-furcated one in H1 could be attributed to a higher tendency of mutations occurring in the latter to revert to an earlier ancestor.

An important point to be noted is that the relatively small number of samples included in our analysis might have biased the shape of the trees for both subtypes. However, repeating the phylogenetic analysis with a larger number of Japanese strains, from this study and from the database, led to more branching or building up on some of the branches, but the tree retained its general structure (data not shown). This could be due to the fact that the main trunk of the tree usually depicts the pathway of advantageous mutations that were fixed by natural selection in both subtypes [24], and thus adding more sequences might capture more variants within each season but would not affect the general number of retained (advantageous) mutations.

The average non-synonymous-to-synonymous substitution ratios (d*N*/d*S*) for the HA1 of H3N2 and H1N1 viruses in this study did not surpass 1 under any of the models allowing for positive selection. Hence, the HA1 subunit is generally under purifying selection, which lowers the frequency of mutations that impose a negative effect on the fitness of the virus, and only certain sites are affected by adaptive selection. The model M3 (discrete) provided the best fit to the data and suggested five sites (50, 131, 144, 145, and 220) in the HA1 subunit of H3N2 under positive selection at a 95% level. Only residue 145 was previously reported to be positively selected [4], and all of the other identified sites were novel and, except site 50, belonged to the antigenic sites of H3.

In the case of H1N1, we found four novel residues (144, 149, 163, and 190) in the HA1 subunit being positively selected, and two of them (residues 163 and 190) belonged to antigenic sites [7]. A previous study of H1N1 isolates from New York and New Zealand between 1994 and 2005 found no evidence of positively selected residues in the HA1 of H1N1 [43]. These results suggested that positively selected sites might change over different periods of time or in different host populations. Notably, positively selected amino acid position 144 was common to both H3 and H1, though this residue was reported to be within an antigenic site only in H3. The detection of positively selected sites that did not belong to already known antigenic sites [7, 39] raises the need for future studies to elucidate a possible antigenic role for these sites.

Remarkably, positively selected residues, 144 in H3 and 163 in H1, were subject to a gain or loss of *N*-glycosylation

during the study period. An important function of *N*-linked glycosylation of influenza virus proteins is to evade detection by the immune system. The loss or gain of *N*-glycosylation sites is an important mechanism underlying antigenic drift through masking or unmasking of the antigenic sites [1, 36]. Our analysis of the HA1 protein of the H3N2 viruses in this study predicted 9–11 potential *N*-glycosylation sites. In the 2000/2001 season, H3N2 viruses that closely clustered with A/Panama/2007/99 acquired an additional *N*-glycosylation site at the HA1 amino acid 144 (I144N) within antigenic site A. However, this site was lost in the following season due to a point mutation (N144D), which resulted in the discontinuity of this lineage and re-emergence of strains with 144N in the 2002/2003 season. This glycosylation site was further retained until the 2006/2007 season, when the dominantly circulating adamantine-resistant viruses lost it again. Smith et al. [34] reported that a single substitution at amino acid position 145 (N145K) caused a significant antigenic cluster transition. The addition of an oligosaccharide chain to the neighboring site (residue 144) is also expected to have a large impact on antigenicity. These findings suggest an important antigenic role for this site, which was also found to be under selective pressure, and which the H3N2 viruses seemed to use to escape detection by the immune system. Notably, an additional *N*-glycosylation site (S45N) was exclusively detected in all the amantadine-sensitive H3N2 strains isolated in the 2006/2007 season. This site has not yet been reported to possess any antigenic role, and it is therefore unclear, whether it could provide any fitness advantage for the amantadine-sensitive lineage.

In the case of H1N1, the seven predicted *N*-glycosylation sites were retained in all strains except in the 2001/2002 season, when some circulating viruses lost one of these sites due to an amino acid replacement N163T or S, antigenic site Sa. This lineage failed to prevail in the following season, and later viruses again retained *N*-glycosylation on this site. These data highlight the importance of *N*-glycosylation in the evolution of influenza A viruses, and especially those occurring within antigenic or positively selected sites should be considered when choosing vaccine strains.

Furthermore, to gain better insight into the evolutionary patterns observed in the HA1 phylogenies of influenza H3N2 and H1N1, we calculated their nucleotide substitution rates in this study. The evolution rate of the H3N2 viruses was found to be slightly faster than that of the H1N1 viruses (4.58×10^{-3} vs. 3.83×10^{-3} nucleotide substitutions/site per year). This difference was intensified by the higher fixation rate of non-synonymous mutations (dN/dS) of HA1 in H3N2 than in H1N1 (0.439 vs. 0.286, respectively), explaining the faster drift in H3N2 viruses. To test whether our results are solely an artifact due to the use of more H3N2 sequences in the analysis, we repeated our test with a smaller number of H3N2 sequences ($n = 60$), and again, a higher rate of substitution for H3N2 was obtained (data not shown). Thus, influenza A/H1N1, as an older subtype, evolves more slowly than the relatively newly emergent A/H3N2, probably due to better adaptation of the former to the human host [24]. This could also be seen in the higher number of mutations observed in the HA1 of H3. The higher variation of HA genes in H3N2 viruses could explain the dominant circulation of these viruses in this study as well as in other reports [11]. Nevertheless, dominant circulation might in turn impose more selective pressure on H3N2 viruses to mutate.

In general, our estimated evolution rates for both H3N2 and H1N1 fell within the range of RNA viruses including influenza [13, 16, 19], although it was more than twice as high as that reported recently for a global collection of H3N2 viruses during 1997–2005 [48]. To account for any differences in that period or in methodologies, we analyzed the evolution rate for a global collection of viruses obtained from the Influenza Virus Resource database (http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html) from a period corresponding to our study. Again, the global evolution rates were lower in comparison with those in our study (data not shown). This supports the notion that viral phylogenies constructed from biased sampling of global isolates might mask the complex dynamics underlying influenza virus evolution within discrete populations, maybe because most influenza virus sequencing done as a part of surveillance programs is focused on detection of serologically novel strains [25]. Future studies of evolution rates of viruses obtained in one fixed area, ideally the tropics, where epidemics occur throughout the year, are warranted for better understanding of evolutionary dynamics of influenza.

The study of the phylodynamics of influenza viruses and its underlying mechanisms is fundamental for understanding how these viruses evolve in response to host immunity and vaccination [24]. In conclusion, we demonstrated that faster evolution and larger diversity of H3N2 viruses in comparison with that of H1N1 is mainly attributable to a higher fixation rate of non-synonymous mutations in the former. We show, for the first time, evidence of the presence of positively selected sites in H1N1 (sites 144, 149, 163, and 190). We also report four novel sites in H3N2 (sites 50, 131, 144, and 220) being under selective pressure. *N*-glycosylation at positively selected sites (e.g. residue 144 in the H3) seemed to contribute to improved viral immune evasion. At a threshold of less than 90%, more sites were found to be under selective pressure (data not shown). These sites may change to strongly selected positions over time through continuous evolution of influenza virus due to host immunological pressure. Therefore, future monitoring of changes in these

sites, especially those that could lead to loss or gain of *N*-glycosylation, might provide potentially important information on which variants might dominate in future epidemics.

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Tohoku J. Exp. Med., 2008, **214**, 113-120

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

Maki Sato,[1,2] Reiko Saito,[1] Isamu Sato,[3] Naohito Tanabe,[1] Yugo Shobugawa,[1] Asami Sasaki,[1] Danjuan Li,[1] Yasushi Suzuki,[1] Mizuho Sato,[1,2] Takatsugu Sakai,[1] Taeko Oguma,[1,2] Hiroki Tsukada,[2] Fumitake Gejyo[2] and Hiroshi Suzuki[1]

[1]Department of Public Health, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan
[2]Department of Medicine II, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan
[3]Yoiko Pediatric Clinic, Niigata, Japan

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

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

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Correspondence: Reiko Saito, M.D., Ph.D., Department of Public Health, Niigata University Graduate School of Medical and Dental Sciences, 1-757, Asahimachi-dori, Chuo-Ward, Niigata City, Niigata 951-8510, Japan.
e-mail: jasmine@med.niigata-u.ac.jp

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

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

Materials and Methods

Study population and laboratory methods

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

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

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

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

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

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

Effectiveness of oseltamivir

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

Statistical analysis

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

RESULTS

Patient characteristics

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

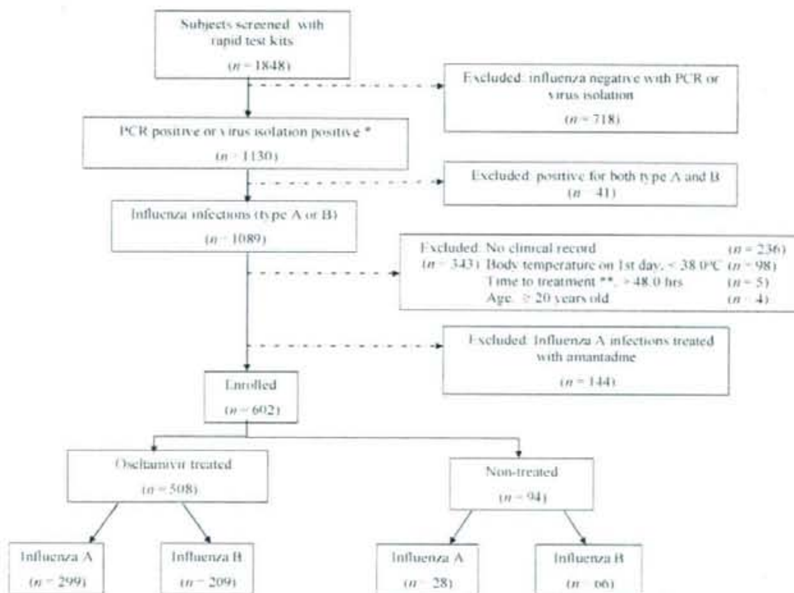

Fig. 1. Flow of participants through the study.
* Subjects were included regardless of rapid test results.
** Time until treatment, the time from the onset of fever to the first dose of treatment.

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

Effectiveness of oseltamivir treatment for influenza A and B

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

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

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

Numbers are mean ± S.D. or n (%).

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

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

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

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

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

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

n.s., not significant.

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

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

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

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

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

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

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

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

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

DISCUSSION

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

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

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

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

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

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

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

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

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

Yugo Shobugawa,[1] Reiko Saito,[1] Isamu Sato,[2] Danjuan Li,[1] Yasushi Suzuki,[1] Asami Sasaki,[1] Maki Sato[1] and Hiroshi Suzuki[1]

[1]Department of Public Health, Graduate School of Medical and Dental Sciences, Niigata University, Niigata, Japan
[2]Yoiko Pediatric Clinic, Niigata, Japan

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

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Correspondence: Yugo Shobugawa, Department of Public Health, Graduate School of Medical and Dental Sciences, Niigata University, 1-757, Asahimachi-dori, Niigata City, Niigata 951-8510, Japan.
e-mail: yugo@med.niigata-u.ac.jp

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

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

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

Materials and Methods

Study population and clinical samples

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

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

Virus isolation and amantadine susceptibility test

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

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

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

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

Analysis of fever reduction

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

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

Statistical analysis

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

RESULTS

Amantadine-sensitive influenza A cases

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

Demographic details of the study groups

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

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

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