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Original article

Surveillance for neuraminidase-inhibitor-resistant influenza viruses in Japan, 1996–2007

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Background: High usage of the neuraminidase inhibitor (NAI) oseltamivir in Japan since 2003 led the Neuraminidase Inhibitor Susceptibility Network to assess the susceptibility of community isolates of influenza viruses to oseltamivir and zanamivir.

Methods: Isolates were tested by the enzyme inhibition assay and by neuraminidase (NA) sequence analysis.

Results: Among 1,141 A(H3N2) viruses and 171 type B viruses collected in Japan during the 2003–2004 season, 3 (0.3%) A(H3N2) isolates showed high 50% inhibitory concentrations (IC_{50}) to oseltamivir. Each possessed a known resistance NA mutation at R292K or E119V. During the 2004–2005 season, no resistance was found among 567 influenza A(H3N2) or 60 A(H1N1) isolates, but 1 of 58 influenza B isolates had an NAI resistance mutation (D197N). Sequence analysis found that 4 (3%) of 132 A(H1N1) viruses from 2005–2006 had known NA resistance mutations (all H274Y), but no additional resistant isolates were detected from that or the subsequent 2006–2007 season. Concurrent testing of a selection of 500 influenza B viruses from 2000 to 2006 showed significant variations between seasons in both oseltamivir and zanamivir IC_{50} values, but no persistent increases over this period.

Conclusions: Our findings suggested possible low-level transmission of resistant variants from oseltamivir-treated patients in several seasons in Japan but no sustained reductions in NAI susceptibility or consistently increased frequency of detecting resistant variants for any strain or subtype, despite high levels of drug use. In particular, although oseltamivir-resistant A(H1N1) viruses with the H274Y mutation spread globally in 2007–2008, we found little evidence for increasing levels of resistant A(H1N1) variants in Japan in preceding years.

Introduction

Annual epidemics of human influenza A and, more variably, influenza B virus infections cause significant amounts of morbidity, economic losses and mortality in the general population [1]. Among recently circulating influenza A viruses, those of the H3N2 subtype have been particularly associated with increases in both morbidity and mortality. In addition, when pandemic influenza occurs due to emergence of a novel type A influenza virus [2], the effects on public health and the economy might be far beyond that of annual epidemics. Immunization of susceptible populations is the principle strategy for protection against both epidemic and pandemic influenza. Two classes of antiviral agents are also available for prevention and treatment of influenza, namely, the adamantanes or M2 ion channel blockers, amantadine and rimantadine, and the neuraminidase inhibitors (NAIs), zanamivir and oseltamivir. In the setting of a major influenza epidemic or pandemic for which vaccine is unavailable, antivirals could reduce morbidity and mortality, given that sufficient amounts were available and deployed rapidly for use [2–4]. Many countries have

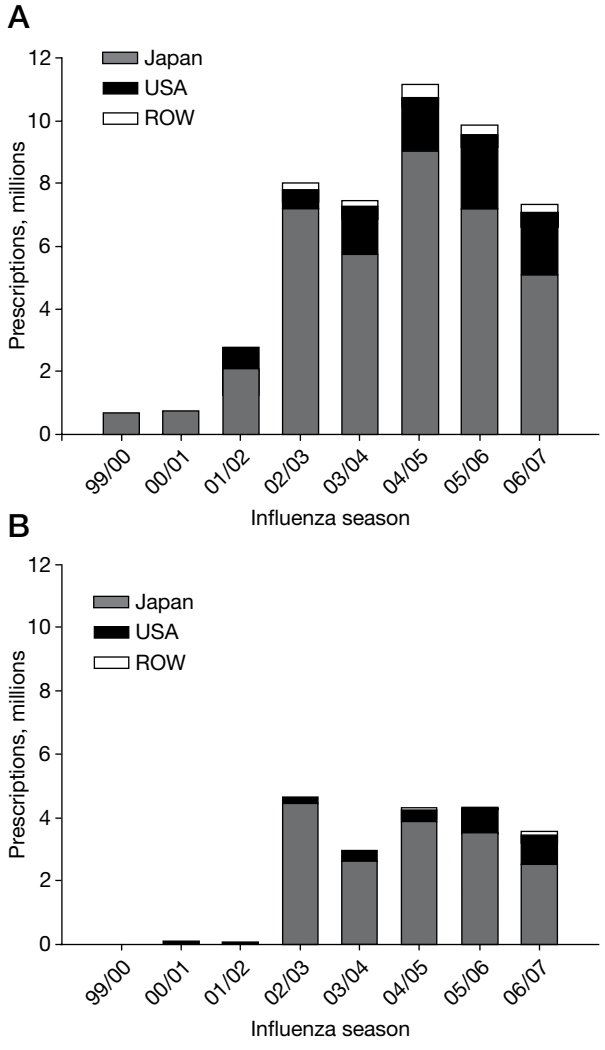
created reserves of NAIs, particularly oseltamivir and sometimes zanamivir, and less often of adamantanes for this purpose, and some have used them in the context of the current H1N1 pandemic.

One of the key concerns with regard to wide-scale use of influenza drugs in the population is the emergence of antiviral drug resistance. Amantadine and rimantadine are effective only against type A influenza viruses, and fully transmissible resistant variants emerge readily during therapeutic use [5,6]. Furthermore, beginning with the 2003–2004 season in the northern hemisphere, global circulation of resistant A(H3N2) variants harbouring a Ser31Asn mutation in the M2 protein has rendered this class of antivirals unreliable [7,8]. More recently, adamantane-resistant A(H1N1) variants with this same mutation have also been increasingly detected [9,10] and the current pandemic H1N1 strain already exhibited adamantane resistance when it was first detected in humans [11].

These resistant variants have circulated in the absence of continuing selective drug pressure and appear at least as transmissible as susceptible wild-type viruses.

The NAIs are inhibitory against both type A and B influenza viruses and are associated with less frequent detection of drug-resistant variants in treated individuals than are the adamantanes [12,13]. Unlike adamantane-resistant variants, those resistant to NAIs usually appear to be biologically impaired in laboratory studies [14,15]. Recent monitoring of NAI susceptibility in community isolates of influenza viruses by the Neuraminidase Inhibitor Susceptibility Network (NISN) found no significant increase of circulating NAI-resistant viruses during the first 3 years after their licensure [16]. However, the use of oseltamivir has increased dramatically in Japan since its licensure in 2001 and during the 2004–2005 season reached the highest levels of population coverage documented to date (Figure 1). Furthermore, the frequency of detecting drug-resistant mutants in oseltamivir-treated children appears to be higher than previously reported [17,18]. These circumstances led NISN to undertake surveillance for NAI susceptibility among community epidemic isolates collected during the 2003–2007 influenza seasons in Japan, in order to determine whether increased oseltamivir use was associated with emergence and transmission of NAI-resistant variants in the community. The results reported here, as well as those from 3 years surveillance carried out previously by the NISN [16,19], suggest that there was evidence for some community transmission of oseltamivir-resistant variants, including oseltamivir-resistant A(H1N1) viruses during the 2005–2006 season. However, despite the global emergence of oseltamivir resistant A(H1N1) viruses in 2007–2008 [20,21], there was no evidence of these resistant viruses circulating in Japan in the preceding 2006–2007 season and no evidence of sustained decreases in susceptibility to NAIs in influenza A or B viruses over this period.

Figure 1. Oseltamivir prescriptions in Japan and other countries, 1999–2007



(A) Total number of oseltamivir prescriptions in Japan, USA, and remainder of world (ROW) from 1999 through the 2006–2007 season. **(B)** Prescriptions for paediatric use [27].

Methods

Viruses and cells

Clinical isolates of influenza A and B viruses were recovered from ambulatory and hospitalized patients by sentinel clinics and hospitals from September 2003 to June 2007. The specimens were collected before the patients were prescribed any drugs. Although none of the isolates came from patients known to have received oseltamivir treatment, it was not possible

Table 1. Numbers of influenza viruses isolated in Japan during the 2003–2007 influenza seasons and received by the WHO CC, NIID

Type/subtype	Season			
	2003–2004	2004–2005	2005–2006	2006–2007
A/H1N1	5	184	1347	576
A/H3N2	4,800	2,531	3,401	2,287
B[a]	291 (1)	3,359 (1)	519 (100)	1,987 (99)
Total	5,096	6,074	5,267	4,850

Data are numbers of viruses unless indicated otherwise. [a]Data are numbers of influenza type B viruses and percentage of B/Victoria lineage. WHO CC, NIID, World Health Organization Collaborating Center for Reference & Research on Influenza, National Institute of Infectious Diseases (Tokyo, Japan).

Table 2. Testing methods used for detecting resistance and determining susceptibilities to NAIs

Year	NISN		WHO CC, NIID		
	Primary screen[a]	Confirmation of resistance	Primary screen	Confirmation of resistance	Influenza B time series
Baseline 1996–1999 [19]	CL NAI	Sequencing	ND	–	–
1999–2000 [16]	CL NAI	Sequencing	ND	–	–
2000–2001 [16]	CL NAI	Sequencing	ND	–	FL NAI
2001–2002 [16]	CL NAI	Sequencing	ND	–	FL NAI
2002–2003	ND		ND	–	FL NAI
2003–2004	CL NAI	Sequencing	Sequencing	CL NAI	FL NAI
2004–2005	CL NAI	Sequencing	Sequencing	CL NAI	FL NAI
2005–2006	ND	–	Sequencing	CL NAI	FL NAI
2006–2007	ND	–	Sequencing	CL NAI	ND

[a]Primary screening by Viromed Laboratories (Minnetonka, MN, USA), except for influenza B 2004–2005, carried out at CSIRO (Melbourne, Australia). CL NAI; NA-Star chemiluminescent neuraminidase inhibition assay; FL NAI, MUNANA-based fluorescent neuraminidase inhibition assay; NAI, neuraminidase inhibitor; ND not done; NISN, Neuraminidase Inhibitor Susceptibility Network; WHO CC, NIID, World Health Organization Collaborating Center for Reference & Research on Influenza, National Institute of Infectious Diseases (Tokyo, Japan).

to determine whether the patients were exposed to contacts on oseltamivir treatment. The viruses used in this study were provided to the National Institute of Infectious Diseases (NIID; Tokyo, Japan) by municipal and prefectural public health institutes across Japan. A random selection of approximately 10% of the original isolates were used for these studies. The total number of isolates and the relative frequency of influenza A(H1N1), A(H3N2) and B viruses tested varied from across the seasons (Table 1). Typing and subtyping was done using a haemagglutination inhibition assay. In addition, sequence analysis of the *HA* and *NA* genes was done for almost all viruses received by the NIID.

Initial isolation was performed in Madin–Darby canine kidney (MDCK) cells. For expansion of virus isolates for NAI assay, MDCK cells were subcultured in phenol red-free Eagle's minimum Essential medium (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; Summit Biotechnology, Fort Collins, CO, USA), 1 mM L-glutamine, 1% HEPES and 1% penicillin-streptomycin (all from Gibco). Virus growth medium containing 0.14% bovine serum albumin fraction V instead of FBS, and 2.5 μg/ml of tosyl-sulfonyl phenylalanyl chloromethyl ketone-trypsin (Worthington Biochemical Co., Lakewood, NJ, USA) was used for virus growth. Initial isolation was done by NIID and expansion of isolates was performed at Viromed Laboratories (Minnetonka, MN, USA).

Drugs and NAI susceptibility assays

Several different assay methods were used to test susceptibility and confirm resistance (Table 2). In all assays, samples were all pre-titrated prior to the inhibition assay, to determine an amount of virus to use that was in the linear portion of the activity curves. Susceptibility to oseltamivir carboxylate and zanamivir was examined initially in a previously described chemiluminescent (CL) enzyme inhibition assay [19,22], using neuraminidase (NA)-star (Tropix, Bedford, MA, USA) as the substrate [23]. Between 1996 and 2005, screening assays were performed by Viromed Laboratories on masked samples, and the values were reported as the drug concentrations required to inhibit enzyme activity by 50% (IC_{50}). Oseltamivir carboxylate, the active compound of the ethyl ester prodrug oseltamivir phosphate, was supplied to Viromed Laboratories by Noel Roberts (Roche Products, Ltd, Welwyn Garden City, UK). Zanamivir was provided by Margaret Tisdale (GlaxoSmithKline, Stevenage,

UK). Control viruses with documented oseltamivir susceptibility or resistance caused by recognized NA mutations were included in assay runs. For type B viruses, a B/Memphis/20/96 isolate with an R152K NA mutation was used [24] and for type A viruses an A/Wuhan/359/95 (H3N2)-like isolate with an E119V NA mutation, and an A/Sydney/5/97(H3N2)-like isolate with an R292K NA mutation were used.

Because of resource limitations, isolates from the 2003–2004 season were tested routinely only for oseltamivir susceptibility. In the 2004–2005 season, samples were screened against both drugs. However, initial testing of the influenza B isolates revealed an unexpectedly high number of isolates with increased IC_{50} values, and an increase in the mean IC_{50} [25]. Independent testing of 58 influenza B isolates from 2004–2005 was carried out using the NA-star CL assay (CL NAI; Applied Biosystems, Foster City, CA, USA) in the laboratory of Jennifer McKimm Breschkin (Commonwealth Scientific and Industrial Research Organization, CSIRO, Melbourne, Australia).

In addition to the phenotypical data from the NAI assays, the frequency of known resistance mutations was also determined by analysis of sequence data from the World Health Organization Collaborating Center for Reference and Research on Influenza in the NIID and from the National Institute of Technology and Evaluation (NITE) laboratories (Tokyo, Japan; Table 2) for isolates from seasons 2003–2004 to 2006–2007. Viral isolates were analysed for known NAI resistance mutations and samples with amino acid mutations suggesting resistance were confirmed by testing in the CL NAI assay at the NIID. Sequencing of the *NA* and *HA* genes was carried out according to the standard manufacturer's protocols (Applied Biosystems, Boston, MA, USA). Confirmatory sequencing of resistant isolates from 2003–2004 and 2004–2005 was also carried out at the Health Protection Agency (London, UK) and at the Influenza Division, Centers for Disease Control and Prevention (Atlanta, GA, USA).

Time-series study for influenza B susceptibility

The drug sensitivity of 500 randomly selected influenza B isolates from 2000–2006 was tested against both oseltamivir carboxylate (provided by Keith Watson, Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia) and zanamivir (provided by Margaret Tisdale, GlaxoSmithKline) to determine whether any significant changes in IC_{50} values were seen in isolates from different years tested in the same assay. Each assay batch contained comparable proportions of isolates from every season. These assays were conducted with the MUNANA-based fluorescent (FL) NAI assay (Table 2) [26], in the laboratory of JLMB.

Data analyses

Previously described methods were used to identify isolates with high IC_{50} values for further phenotypical testing and sequence analysis [16]. Because IC_{50} values are not normally distributed, values underwent $\log_{10}$ transformation for analysis. Robust data analyses (box and whisker plots) were used to identify extreme IC_{50} values. Two types of outliers were defined, mild (between 1.5 and 3.0× the interquartile range from the 25th and 75th percentiles) and extreme (>3.0× the interquartile range from the 25th and 75th percentiles and at least 10-fold higher than the mean IC_{50}). All known resistant variants were extreme outliers.

To assess whether changes in the NAI susceptibility among influenza A(H3N2) and A(H1N1) or B viruses had occurred since the introduction of the drugs into Japan, we also compared the results with those obtained from our previous analysis of isolates during the influenza seasons from 1996 to 2003 [16,19]. The subsets of Japanese data were taken from the total data sets used in the prior publications for the 3 years before (1996–2000) [19] and after (2000–2002) [16] approval of the drugs in Japan.

Results

Neuraminidase inhibitor usage in Japan

In Japan, the capsule form of oseltamivir is prescribed for adult and juvenile patients with a body weight ≥37.5 kg, and the paediatric suspension syrup can be prescribed in children as young as 1 year old. As shown in Figure 1A, the estimated number of oseltamivir treatment courses has been increasing since its introduction in February 2001 [27]. Overall, >6 million treatment courses in Japan were prescribed during the 2003–2004 season and >10 million during the following season. Approximately 70% of this amount was oseltamivir capsules and approximately 30% was the paediatric suspension (Figure 1B) [27]. Zanamivir use was much less during this period and averaged <200,000 courses annually.

Virus isolation patterns

During the 2003–2004 influenza season, >90% of isolates characterized at municipal public health institutes were A(H3N2) subtype (Table 1). A(H3N2) viruses represented >40% of all isolates through each subsequent season through 2006–2007. Levels of influenza A(H1N1) and B activities were more variable with a higher incidence of A(H1N1) compared with influenza B in 2005–2006, whereas influenza B was more prevalent in all other seasons. There was a change observed in the predominant influenza B lineage from almost exclusively B/Yamagata lineage in the 2003–2005 seasons to B/Victoria lineage in the 2005–2007 seasons.

Table 3. The 50% inhibitory concentrations in NA-star-based chemiluminescent enzyme inhibition assays for influenza A(H1N1,H3N2) and influenza B viruses isolated in Japan, 1996–2005

	Oseltamivir						Zanamivir					
Type/subtype	Baseline 1996–1999[a]	1999–2000[b]	2000–2001[b]	2001–2002[b]	2003–2004	2004–2005	Baseline 1996–1999[a]	1999–2000[b]	2000–2001[b]	2001–2002[b]	2003–2004	2004–2005
A/N1												
Isolates tested, *n*[c]	ND	15	75	124	ND	60	ND	15	75	124	ND	60
Median IC_{50} value (range)	ND	1.08 (0.62–1.61)	0.57 (0.08–1.91)	0.51 (0.18–69.18)	ND	1.29 (0.01–5.71)	ND	0.70 (0.48–5.37)	0.46 (0.09–1.41)	0.23 (0.10–151.36)	ND	1.23 (0.13–5.91)
Mean IC_{50} value (±SD)	ND	1.09 (1.26)	0.49 (2.07)	0.56 (1.94)	ND	1.01 (3.33)	ND	0.80 (1.73)	0.42 (1.70)	0.25 (1.96)	ND	1.10 (2.06)
Extreme, *n*[d]	ND	0	0	1	ND	0	ND	1	0	1	ND	0
Mild, *n*[e]	ND	0	0	4	ND	0	ND	0	3	0	ND	1
A/N2												
Isolates tested, *n*[c]	7	43	37	88	1,141	562	7	43	37	88	ND	567
Median IC_{50} value (range)	0.98 (0.57–1.85)	0.54 (0.17–2.02)	0.50 (0.01–0.76)	0.44 (0.08–1.35)	0.81 (0.07–9,600)	1.37 (0.02–31.52)	2.78 (1.18–4.22)	1.87 (0.61–5.37)	1.21 (0.08–2.86)	0.95 (0.16–3.11)	ND	2.25 (0.03–25.62)
Mean IC_{50} value (±SD)	0.99 (1.46)	0.56 (1.60)	0.38 (2.15)	0.44 (1.60)	0.83 (2.30)	1.28 (2.94)	2.35 (1.54)	1.77 (1.44)	1.05 (2.39)	0.86 (1.65)	ND	1.93 (2.84)
Extreme, *n*[d]	0	0	0	0	3	0	0	0	0	0	ND	0
Mild, *n*[e]	0	1	0	0	12	1	0	2	0	0	ND	1
B												
Isolates tested, *n*[c]	4	14	93	146	171	58	4	14	93	146	ND	58
Median IC_{50} value (range)	5.66 (4.83–7.88)	7.97 (4.73–17.47)	11.42 (1.93–47.66)	2.24 (0.43–14.03)	12.40 (1.98–60.66)	4.86 (1.49–18.80)	2.76 (2.06–4.09)	2.12 (1.29–4.29)	1.99 (0.48–9.91)	0.98 (0.18–2.05)	ND	3.11 (1.72–11.13)
Mean IC_{50} value (±SD)	5.91 (1.25)	8.25 (1.55)	11.35 (1.98)	2.43 (1.90)	13.85 (1.74)	4.94 (1.55)	2.83 (1.36)	2.37 (1.41)	2.17 (2.14)	0.95 (1.45)	ND	3.19 (1.40)
Extreme, *n*[d]	0	0	0	0	0	0	0	0	0	0	ND	0
Mild, *n*[e]	0	0	0	1	8	2	0	0	0	0	ND	3

Statistical analyses were performed on log_{10} transformed values of all isolates in each grouping, and the results were then back-transformed. Isolates were collected between October 1 of the first year and September 30 of the second year. Data are nM. [a]Japanese subset from McKimm-Breschkin *et al.* [19]. [b]Japanese subset from Monto *et al.* [16]. [c]Data are number of isolates tested. [d]Number of extreme outliers. [e]Number of mild outliers. IC_{50} 50% inhibitory concentraion; NA, neuraminidase; ND, not done.

NAI susceptibility testing

NAI susceptibility data from the seasons beginning in 1996 and extending through the 2004–2005 season are shown in Table 3. Data for the 2002–2003 season in Japan was not available, and susceptibility testing for zanamivir was not performed during the 2003–2004 season.

2003–2004 Season

A total of 1,141 A(H3N2) and 171 B viruses isolated during the 2003–2004 influenza season were available for testing by CL NAI assay. The median oseltamivir carboxylate IC_{50} values for A(H3N2) and B viruses were 0.8 nM and 12.4 nM, respectively (Table 3). Among the A(H3N2) viruses examined, 3 (0.3%) viruses were recognized as extreme outliers: A/Okayama/23/2004, A/Tokyo/2101/04 and A/Fukui/45/2004. Both A/Tokyo/2101/04 and A/Fukui/45/04 were fully susceptible to zanamivir in the NAI assay (Table 3). Independent testing in NISN member laboratories with the CL NAI assay found that the A/Okayama/23/04 isolate had IC_{50} values of 2,000–10,000 nM and 10–20 nM for oseltamivir carboxylate and zanamivir, respectively, whereas the A/Tokyo/2101/04 and A/Fukui/45/04 only had increased IC_{50} values of 5–10 nM to oseltamivir.

Twelve other A(H3N2) viruses were categorized as mild outliers and had oseltamivir carboxylate IC_{50} values that ranged from 4.0 to 5.7 nM; the IC_{50} values for these isolates were also <10-fold above the median value for all A(H3N2) viruses. Among 171 B isolates, none were extreme outliers; 8 viruses with IC_{50} values from 49.2 to 60.7 nM were categorized as mild outliers.

NA amino acid sequences were determined for the extreme outliers of the A(H3N2) viruses, as well as for 5 mild outliers and 6 other isolates of A(H3N2) viruses and for 8 mild outliers of influenza B viruses. None of the mild outliers had mutations in their NA genes known to be associated with oseltamivir resistance. By contrast, previously recognized nucleotide substitutions and associated amino acid substitutions conferring oseltamivir resistance [13] were found in two extreme outliers: A/Okayama/23/04 had a catalytic site substitution, R292K, whereas A/Tokyo/2101/04 and A/Fukui/45/04 had a framework substitution, E119V. No significant changes in the deduced amino acid sequences of the HA were noted in the other three 2003–2004 extreme outliers of A(H3N2) viruses. Sequence analysis, by the Japanese laboratories of the NAs of 1,180 isolates, including the 1,141 studied here, identified only the same three resistant viruses.

2004–2005 Season

A total of 60 A(H1N1), 567 A(H3N2) and 58 influenza B isolates from the 2004–2005 season were available for testing in the CL NAI assay. The median oseltamivir carboxylate IC_{50} values for A(H3N2), A(H1N1) and B NAs were 1.4 nM, 1.3 nM and 4.9 nM, respectively (Table 3). The median zanamivir IC_{50} values for A(H3N2), A(H1N1) and influenza B NAs were 2.3 nM, 1.2 nM and 3.1 nM, respectively (Table 3). Among the A(H3N2) viruses examined, none were recognized as resistant in the CL NAI assay, and only one was categorized as a mild outlier. Similarly, none of 60 A(H1N1) viruses was found to be resistant. Two of 58 influenza B isolates were mild outliers to both drugs, and sequence analysis identified a known resistance mutation in one of them, namely D197N [25,28]. Sequence analysis of 223 influenza B isolates did not identify any additional isolates with known resistance mutations (Table 4), and testing of a further discrete 97 influenza B isolates in the time series study (FL NAI assay) also did not detect phenotypical resistance (Table 5).

Table 4. Frequency of neuraminidase-inhibitor-resistant viruses isolated in Japan during the 2003–2007 influenza seasons[a]

	Season			
Type/subtype	2003–2004	2004–2005	2005–2006	2006–2007
A/H1N1				
Isolates tested, *n*	0	60	132	54
Resistant, *n* (%)	0	0	4 (3)	0
A/H3N2				
Isolates tested, *n*	1,180	558	250	134
Resistant, *n* (%)	3 (0.3)	0	0	0
B				
Isolates tested, *n*	171	223	61	119
Resistant, *n* (%)	0	1 (0.4)	0	0
Total				
Total isolates tested, *n*	1,351	841	443	307
Total resistant, *n* (%)	3 (0.2)	1 (0.1)	4 (0.9)	0

[a]Neuraminidase (NA) sequences were screened for known resistance mutations by sequencing of the full length NA. Viruses with recognized mutations were confirmed as phenotypically-resistant in the chemiluminescent assay.

2005–2006 Season

Sequence analysis of 250 influenza A(H3N2) and 61 influenza B isolates did not identify any viruses with known resistance mutations (Table 4), nor were any resistant influenza B isolates identified from the additional 58 viruses tested in the time series experiment (Table 5). By contrast, 4 (3%) of 132 A(H1N1) viruses possessed an H274Y (H275Y in N1 numbering system) mutation known to confer high-level oseltamivir resistance in N1-containing viruses [29,30]. These isolates were confirmed as phenotypically resistant to oseltamivir by the CL NAI assay, with IC_{50} values for oseltamivir ranging from 122 to 464 nM and for zanamivir from 2.9 to 6.7 nM. All samples were also subsequently tested in the CL NAI assay by the NIID, but no additional resistant isolates were detected (data not shown).

2006–2007 Season

Although four viruses with the H274Y mutation were identified among the 2005–2006 isolates, no H274Y mutations were detected among the NAs sequenced from 54 A(H1N1) viruses from 2006–2007 (Table 4). Sequence analysis of the NAs from 134 A(H3N2) and 119 influenza B isolates also did not identify any with known mutations. Subsequent testing of all isolates by the NIID in the CL NAI assay did not identify any further resistant isolates.

Time series study for influenza B susceptibility

Our initial CL NAI screening results showed an increase in the mean IC_{50} values for oseltamivir carboxylate for influenza B NAs between the 2001–2002 and 2003–2004 seasons (Table 3), but the testing had been carried in assays performed over multiple years. In order to exclude a possible confounding effect of variance in assay results from year to year, we retested 500 randomly selected influenza B isolates from 2000–2006 in the FL NAI assay. Viruses were tested in batches containing comparable proportions of isolates from each period of interest. The results (Table 5) indicated that there was season to season variation in susceptibilities to both inhibitors. The mean IC_{50} values for oseltamivir carboxylate in 2002–2003 and 2003–2004 were significantly higher than the mean IC_{50} values observed in 2004–2005 and 2005–2006 ($P<0.05$) when compared by orthogonal linear contrast analyses. In addition, there were significant differences in the mean IC_{50} values between 2002–2003 and 2003–2004 and between 2004–2005 and 2005–2006 for zanamivir ($P<0.05$). Although increased IC_{50} values were observed in some years, there was no sustained decrease in susceptibility to either drug over the 6 year period (Table 5). The variations were not related to differences in the proportions of viruses deriving from the B/Yamagata and B/Victoria lineages of influenza B during this time period. For example, increased IC_{50} values to oseltamivir were observed in the 2002–2003 and 2003–2004 seasons when 17% and 87.5% of isolates tested were B/Yamagata lineage viruses, respectively.

Discussion

In recent influenza seasons, Japan has had the highest per capita use of oseltamivir in the world (Figure 1). During each season of our surveillance, 10–12 million patients with influenza-like illness were estimated to have visited clinics. In particular, during the 2003–2004 season, a brisk influenza epidemic, largely caused by circulation of a drift variant influenza A(H3N2) virus (A/Fujian/411/2002[H3N2]-like), was associated with oseltamivir treatment being given to approximately 6 million persons, or approximately 5% of the Japanese population. During the following season, oseltamivir use increased to >10 million courses, or nearly

Table 5. Time series analysis of IC_{50} values of Japanese influenza B isolates for oseltamivir carboxylate and zanamivir in MUNANA-based fluorometric assay from seasons 2000–2006[a]

	Season					
	2000–2001	2001–2002	2002–2003	2003–2004	2004–2005	2005–2006
Oseltamivir						
n	97	94	96	49	97	58
Mean IC_{50} value	25.3	18.7	33.6	34.4	26.9	24.6
95% CI[b]	(23.1–27.7)	(17.0–20.7)	(30.6–36.9)	(30.5–38.9)	(24.6–29.3)	(21.6–28.07)
Zanamivir						
n	98	94	96	49	97	58
Mean IC_{50} value	2.1	1.8	2.6	3.8	4.3	2.2
95% CI[b]	(1.9–2.3)	(1.7–2.0)	(2.4–2.9)	(3.4–4.3)	(3.9–4.8)	(1.9–2.5)
B/Yamagata, %	99.0	39.6	17.0	87.5	97.9	0

[a]Viruses were tested in batches containing comparable proportions of isolates from each time period. [b]Data are 95% confidence interval (CI) estimates of the mean 50% inhibitory concentrations (IC_{50}) measured in nM.

10% of the population. These levels of drug use greatly exceed those anticipated in most countries during pandemic influenza and approach those in many countries that have developed or are developing oseltamivir stockpiles. Increasing use of oseltamivir in Japan, the recent reports of oseltamivir-resistant variants emerging in 16–18% of oseltamivir-treated children [17,18], and evidence for transmissibility of some NAI-resistant variants in animal models [14] and in households [31] raised concerns about the potential appearance and transmission of NAI-resistant viruses in the community at large. Consequently, NISN undertook an investigation of large numbers of community isolates in Japan starting with the 2003–2004 influenza season to look for evidence of oseltamivir resistance emergence and spread at the community level. Our results to 2007 indicate that NAI resistance was encountered at low levels in community isolates, and the overall prevalence of circulating resistant strains recognized in this study does not appear to have increased substantially compared with that obtained in our earlier study encompassing the 1999–2002 seasons in Japan (Table 3) [16].

We found evidence for apparent community transmission of oseltamivir-resistant viruses during several seasons. During the 2005–2006 season, A(H1N1) viruses with the H274Y mutation (H275Y in N1 numbering) that confers high-level oseltamivir resistance accounted for approximately 3% of the A(H1N1) viruses tested. Of note, such oseltamivir-resistant variants were not detected during the subsequent 2006–2007 season, which suggested that oseltamivir use was not fostering more frequent A(H1N1) resistance. Community A(H1N1) isolates with the H274Y mutation appeared widely for the first time in multiple countries in Europe, Asia and North America during the 2007–2008 season [20,32–34], and were subsequently detected at high frequency in the southern hemisphere, including 100% of A(H1N1) isolates in South Africa [20] and in the 2008–2009 winter >98% of H1N1 isolates were resistant.

There is no evidence to indicate this high level of resistance in A(H1N1) viruses arose because of the high use of oseltamivir in Japan. Indeed, although H1N1 viruses were predominant in Japan in 2007–2008, only 45 (2.6%) of a total of 1,734 isolates examined were resistant [20]. Among these resistant H1N1 viruses, one resistant virus (clade 2C) was found in the early phase of that season, which was considered a spontaneous mutant. Subsequently in early 2008, 26 'Hawaii'-lineage-resistant H1N1 viruses of clade 2B were identified. These occurred in 9 prefectures but clustered in 2 prefectures out of the 9 prefectures (10 and 4 viruses, respectively). These viruses might have been introduced into Japan from outside, probably from Hawaii, and spread to an extent in these areas. Finally, during the late 2007–2008 season, 18 'European' lineage mutants were found in 3 prefectures. Although the percentage of the lineage virus was 2.6% of total H1N1 in Japan, it was 32% in a prefecture affected by the virus, and some mutants were also isolated in surrounding prefectures. This indicated that the H1N1 resistant virus was introduced into Japan and spread significantly in a localized area.

The continued circulation of oseltamivir-resistant A(H1N1) viruses has occurred in the apparent absence of selective drug pressure, and their global spread demonstrates that these recent oseltamivir-resistant A(H1N1) with H274Y variants are efficiently transmitted from person to person. Earlier studies of A(H1N1) and A(H5N1) variants with the H274Y mutation reported variable reductions in replication fitness *in vitro* and in ferrets [35,36], although a clinical isolate of an A(H1N1) virus with this mutation was transmissible between ferrets [14] and another A(H5N1) virus with this mutation was able to replicate and cause a lethal infection in mice [37].

To our knowledge, in the current study, the patients with the resistant viruses were not treated with oseltamivir before the specimens were taken for virus isolation. Unfortunately, no further clinical and epidemiological data were available, so that it is unknown whether they might have been exposed to oseltamivir-treated patients. Consequently, the resistant variants possibly represented primary resistance or were transmitted from an oseltamivir-treated patient. No primary drug resistance was recognized in influenza viruses before the introduction of oseltamivir in surveillance studies [19,38] or in large numbers of isolates collected in prospective treatment studies [39–41]. However, we cannot exclude that a low level of resistant variants occurs in circulating viruses in the absence of selective pressure. It is clear, however, that the H3N2 resistant variants were not related to each other because of geographical scattering of the places of isolation (Tokyo, Fukuoka and Okayama). Tokyo is in the centre of Japan, whereas Fukuoka is approximately 1,000 km west and Okayama is located between the two cities. In addition, the three resistant A(H3N2) viruses belonged to at least two different sublineages of NA by phylogenetic analysis.

The more likely explanation is that the resistant viruses found in this study represented transmission from an oseltamivir-treated patient to a close contact. In contrast to the experience observed with the M2 inhibitor rimantadine [5], no resistance emergence or transmission was detected in household-based studies of oseltamivir or zanamivir treatment and post-exposure prophylaxis [42,43]. Animal model studies indicate that oseltamivir-resistant A(H3N2) variants

possessing a R292K substitution are less infectious and transmissible in ferrets, compared with its wild-type parents [14,44]. By contrast, another oseltamivir-resistant A(H3N2) variant caused by E119V substitution is fully replication competent and transmissible in ferrets [14,15,44]. Of note, in the guinea pig model an A(H3N2) virus with this mutation was transmissible by direct contact but not by aerosol, in contrast to the wild-type [45]. Therefore, because no resistance was seen in community isolates prior to the introduction of the NA inhibitors, the detection of viruses in untreated patients with the E119V, R292K and H274Y mutations would be more consistent with transmission from an oseltamivir-treated contact in whom the resistance arose, rather that from spontaneous emergence in the untreated patient.

In addition to screening for resistant variants, our studies also looked for changes in oseltamivir susceptibility over time to detect smaller decrements in sensitivity related to its increasing use. For influenza A(H3N2) and A(H1N1) viruses we found no substantial changes in susceptibility over time, following introduction of the NAIs into clinical practice to 2005 (Table 3). For influenza B viruses, we confirmed their lower susceptibility to oseltamivir compared with influenza A viruses [16,19], but also noted some season-to-season variation in IC_{50} values for both oseltamivir carboxylate and zanamivir (Table 5). Recent studies from Japan [46] and the United Kingdom (MZ, unpublished observations) reported some season-to-season variation in oseltamivir susceptibility among community isolates of influenza B viruses, but no sustained pattern of decreased susceptibility to NAIs . Another study of predominately North American B isolates found no important differences in oseltamivir IC_{50} values across three seasons from 2004–2007 [34]. Of note, the Japanese study [46] used an FL NAI assay, in which the influenza B NAs are known to have higher IC_{50} values compared with the CL NAI assay [19,22]. Their reported mean IC_{50} for oseltamivir was 75.4 nM for 193 influenza B isolates, or approximately 250-fold less susceptible to oseltamivir compared with influenza A(H3N2) viruses. This compares to a mean IC_{50} in our time series of 25–35 nM for oseltamivir carboxylate and of 2–4 nM for zanamivir (Table 5). Various parameters including buffer, pH and substrate concentration can affect the IC_{50} (JLMB, unpublished observations) [47], which could account for their higher IC_{50} compared with those obtained here. Of note, the lower susceptibility of influenza B viruses seen in both the FL and CL NAI assays relative to influenza A NAs might account for the slower clinical and virological responses to treatment in young children [46] and prophylaxis failures in immunocompromised hosts [28]. Continued monitoring of influenza B susceptibility is therefore essential.

In the setting of a pandemic or major epidemic caused by a virus with a poor antigenic match to available vaccines, NAIs could play an important role in the initial response until sufficient vaccines were available [2–4]. Currently, many countries are developing stockpiles of oseltamivir and other antivirals for such a threat. Given first exposure to a novel virus, the emergence of resistant viruses from NAI-treated patients might be expected to be higher in an immunologically naive population than observed in the interpandemic period in those with some degree of pre-existing immunity. The frequencies of resistance emergence in young children might be more indicative of those expected in pandemic disease, and these have been up to 80% for amantadine [48] and 16–18% for oseltamivir [17,18] in paediatric studies to date. Zanamivir resistance emergence in this target population has not been adequately examined, in part because the current Diskhaler device cannot be reliably used below age 5 years. The principle public health concerns are whether such variants are readily transmissible and capable of causing disease. Both household and, more recently, global transmission [7–10] of amantadine-resistant A(H3N2) and, to a lesser extent, A(H1N1) variants have been documented. Although we found that the risk of resistance emergence appears to be less with oseltamivir use, the recent experience with widespread circulation of oseltamivir-resistant A(H1N1) viruses since the 2007–2008 season illustrates the unpredictability of influenza virus epidemiology [20,21,32,33]. One mathematical modelling study [49] predicted little community spread of NAI-resistant variants when they were associated with small decrements in transmissibility, and the importance of transmission fitness as a risk factor in spread of resistant variants has been confirmed in other models of pandemic influenza [50,51]. Thus, continued surveillance during both epidemic and pandemic periods is crucial to monitor for the transmission of NAI-resistant viruses in order to understand optimal usage of antivirals and to make informed decisions regarding diversification and deployment of stockpile contents.

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