Antiviral Research 99 (2013) 261–269

Contents lists available at SciVerse ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Neuraminidase inhibitor susceptibility profile of pandemic and seasonal influenza viruses during the 2009–2010 and 2010–2011 influenza seasons in Japan

Clyde Dapat [a,*], Hiroki Kondo [a], Isolde C. Dapat [a], Tatiana Baranovich [a,1], Yasushi Suzuki [a,2], Yugo Shobugawa [a], Kousuke Saito [a], Reiko Saito [a], Hiroshi Suzuki [b]

[a] Division of International Health (Public Health), Graduate School of Medical and Dental Sciences, Niigata University, Niigata, Japan
[b] School of Nursing, Niigata Seiryo University, Niigata, Japan

ARTICLE INFO

Article history:
Received 21 December 2012
Revised 5 June 2013
Accepted 9 June 2013
Available online 17 June 2013

Keywords:
Influenza
Half maximal inhibitory concentration (IC50)
Oseltamivir
Zanamivir
Peramivir
Laninamivir

ABSTRACT

Two new influenza virus neuraminidase inhibitors (NAIs), peramivir and laninamivir, were approved in 2010 which resulted to four NAIs that were used during the 2010–2011 influenza season in Japan. This study aims to monitor the susceptibility of influenza virus isolates in 2009–2010 and 2010–2011 influenza seasons in Japan to the four NAIs using the fluorescence-based 50% inhibitory concentration (IC_{50}) method. Outliers were identified using box-and-whisker plot analysis and full NA gene sequencing was performed to determine the mutations that are associated with reduction of susceptibility to NAIs. A total of 117 influenza A(H1N1)pdm09, 59 A(H3N2), and 18 type B viruses were tested before NAI treatment and eight A(H1N1)pdm09 and 1 type B viruses were examined from patients after NAI treatment in the two seasons. NA inhibition assay showed type A influenza viruses were more susceptible to NAIs than type B viruses. The peramivir and laninamivir IC_{50} values of both type A and B viruses were significantly lower than the oseltamivir and zanamivir IC_{50} values. Among influenza A(H1N1)pdm09 viruses, the prevalence of H274Y viruses increased from 0% in the 2009–2010 season to 3% in the 2010–2011 season. These H274Y viruses were resistant to oseltamivir and peramivir with 200–300 fold increase in IC_{50} values but remained sensitive to zanamivir and laninamivir. Other mutations in NA, such as I222T and M241I were identified among the outliers. Among influenza A(H3N2) viruses, two outliers were identified with D151G and T148I mutations, which exhibited a reduction in susceptibility to oseltamivir and zanamivir, respectively. Among type B viruses, no outliers were identified to the four NAIs. For paired samples that were collected before and after drug treatment, three (3/11; 27.3%) H274Y viruses were identified among A(H1N1)pdm09 viruses after oseltamivir treatment but no outliers were found in the laninamivir-treatment group ($n = 3$). Despite widespread use of NAIs in Japan, the prevalence of NAI-resistant influenza viruses is still low.

1. Introduction

Neuraminidase inhibitors (NAIs) are currently used for the prevention and treatment of influenza virus infection. NAIs are transition-state analogues that prevent the cleavage of terminal sialic acid residues that is essential for the release and spread of progeny virions (Colman, 1994; Gubareva, 2004). Examples of NAIs include zanamivir, which is administered by inhalation and oseltamivir, which is available as an oral formulation (Moscona, 2005). In Japan, zanamivir and oseltamivir were approved in 1999 and 2000, respectively and two new drugs, peramivir and laninamivir, were approved in 2010 (Sugaya, 2011). The intravenous peramivir and inhaled laninamivir are administered as a single dose (Kohno et al., 2010; Yamashita et al., 2009).

NAIs were designed to bind competitively to the conserved active site of NA based on its structure and certain amino acid mutations at this site may confer resistance to the drugs (Colman, 1994). Global surveillance reports between 1996 and 2007 showed low incidence of viruses with decreased susceptibility to NAIs (McKimm-Breschkin et al., 2003; Monto et al., 2006; Mungall et al., 2004; Sheu et al., 2008; Tashiro et al., 2009). In the 2007–2008 influenza season, high frequency of oseltamivir-resistant influenza A viruses (H1N1) with H274Y mutation was reported

* Corresponding author. Address: Division of International Health (Public Health), Graduate School of Medical and Dental Sciences, Niigata University, 1-757 Asahimachi-dori, Niigata City, Niigata Prefecture 951-8510, Japan. Tel.: +81 25 2272129; fax: +81 25 2270765.
E-mail address: clyde@med.niigata-u.ac.jp (C. Dapat).
[1] Current address: St. Jude Children's Research Hospital, 262 Danny Thomas Place, Memphis, TN, United States.
[2] Current address: National Institute of Animal Health, National Agriculture and Food Research Organization, Tsukuba, Ibaraki, Japan.

0166-3542/$ - see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.antiviral.2013.06.003

in Norway (67%) (Hauge et al., 2009). However, in Japan, the incidence rate of oseltamivir resistance (<2.6%) was low during the 2007–2008 season, despite widespread use of oseltamivir in the country (Baranovich et al., 2010; Sugaya, 2011; Tashiro et al., 2009; Ujike et al., 2010). In the following influenza season (2008–2009), the oseltamivir-resistant A (H1N1) virus was detected worldwide (Besselaar et al., 2008; Dharan et al., 2009; Hurt et al., 2009) and the frequency of oseltamivir-resistant A (H1N1) virus in Japan almost reached 100% (Baranovich et al., 2010; Ujike et al., 2010).

The NAI susceptibility profile changed when the seasonal A (H1N1) virus was replaced with the pandemic 2009 A (H1N1) virus. Global NAI surveillance showed that the frequency of oseltamivir-resistant A(H1N1)pdm09 viruses was <1.5% in the 2009–2010 influenza season (Hurt et al., 2011a; Longtin et al., 2011; Okomo-Adhiambo et al., 2010b; Ujike et al., 2011). However, in early 2011, an increase in the detection of A(H1N1)pdm09 variants with reduced sensitivity to both oseltamivir and zanamivir due to two mutations, H274Y and S246N, in NA was reported in Singapore (10%) and Australia (30%) (Hurt et al., 2011c). Of particular concern is the sustained community transmission of oseltamivir-resistant A(H1N1)pdm09 viruses in Australia between May and September 2011 (Hurt et al., 2011b, 2012). These reports highlight the importance of close monitoring of antiviral susceptibility profile of influenza viruses.

In the present study, we describe the susceptibility of influenza A(H1N1)pdm09, A(H3N2), and type B viruses to four NAIs (oseltamivir, zanamivir, peramivir, and laninamivir) using the fluorescence-based 50% inhibitory concentration (IC_{50}) method in two consecutive influenza seasons (2009–2010 and 2010–2011) in Japan.

2. Materials and methods

2.1. Compounds

Oseltamivir was provided by Roche Products Ltd. (Basel, Switzerland). Zanamivir was obtained from GlaxoSmithKline (Brentford, United Kingdom). Peramivir was provided by Shionogi & Co., Ltd. (Osaka, Japan). Laninamivir was provided by Daiichi Sankyo Co. Ltd. (Tokyo, Japan). All four NAIs were dissolved in distilled water and aliquots were stored at −30 °C until used.

2.2. Clinical samples

Nasopharyngeal swabs were obtained from patients who presented influenza-like symptoms such as sudden onset of fever, cough, or sore throat at 18 medical facilities in eight prefectures in Japan (Hokkaido, Niigata, Fukushima, Gunma, Kyoto, Osaka, Hyogo, and Nagasaki) during the 2009–2010 and 2010–2011 seasons. Patients were screened for influenza using either Quick Ex flu (Denka Seiken, Tokyo, Japan), ESPLINE Influenza A&B–N (Fujirebio,Tokyo, Japan), or Check FluAB (Alfresa Pharma, Osaka, Japan) rapid test kit. Samples were collected from rapid test kit-positive patients during their first clinic visit prior to drug treatment and second visit samples were obtained from patients when they agreed for another sampling after drug treatment. The choice of drug was based on the advice of clinicians and the preference of patients or guardians. Informed consent was obtained from the patient or patient's guardian. This study was approved by the Medical Faculty Ethics Committee of Niigata University.

2.3. Viruses and cells

A/Kyoto/09K328/2009 (wild-type) and A/Kyoto/09K328-2/2009 (H274Y mutant) viruses that were obtained from our previous study were used as controls for A(H1N1)pdm09 viruses in NAI assay (Dapat et al., 2012). A/Fukui/20/2004 and B/Memphis/20/96 were the wild-type controls for subtype H3N2 and type B viruses, respectively (provided by Dr. Takato Odagiri, WHO Influenza Reference Center, National Institute of Infectious Diseases, Tokyo, Japan).

Madin–Darby canine kidney (MDCK) cells were maintained in minimum essential medium (MEM; Sigma, St. Louis, Mo., USA) supplemented with 10% fetal bovine serum (Gibco, Auckland, New Zealand). Clinical samples were inoculated onto MDCK cells and passaged three times to reach sufficient titers for NAI assay. Third passaged viruses were used for the NAI assay. Hemagglutination inhibition (HI) test was done to identify the type and subtype and cycling-probe real-time PCR assay was performed to screen for H274Y oseltamivir-resistant viruses among A(H1N1)pdm09 isolates (Dapat et al., 2012).

2.4. NAI assay

Susceptibility to NAIs was determined in a fluorescence-based IC_{50} method using 2′-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid (MUNANA; Sigma, St. Louis, Mo., USA) as the substrate (Hurt et al., 2004). Representative samples from each prefecture were selected at random. Prior to NAI assay, samples were titrated to obtain a dilution in the linear segment of the NA activity curve. Input viruses were diluted to a final concentration of 25,000 fluorescence unit based on 12.5:1 signal-to-noise ratio (McKimm-Breschkin, et al., 2003). NAI assay was performed by adding 25 μL of each dilution of NAI to each well of a microtiter plate. For type A viruses, the final concentration range of the four NAIs was from 0.02 to 1250 nM and for type B viruses, the final concentration range was from 2 to 626 nM. Twenty-five (25) μL of diluted virus was then added to each well and plates were incubated at 37 °C for 30 min. Fifty (50) μL of MUNANA substrate (final 25 μM) was added to each well and plates were incubated at 37 °C for 60 min. The reaction was stopped by adding 260 μL of 200 mM of sodium carbonate to each well. Fluorescence was measured using a multi-well plate reader TriStar LB 941 (Berthold Technologies GmbH & Co., Bad Wildbad, Germany).

2.5. IC_{50} and statistical analyses

The IC_{50} values were determined using the XLfit 5.0 software (ID Business Solutions Ltd., Surrey, UK) (Monto et al., 2006). The mean IC_{50} value was calculated from two independent experiments in duplicates. Box-and-whisker plot analysis was performed to determine the statistical cutoffs of outliers. A sample is considered a mild outlier if its IC_{50} value is more than three times the interquartile range (IQR) from the 75th percentile and a sample is an extreme outlier if its IC_{50} value is at least 10-fold higher than the mean and more than three times the IQR from the 75th percentile (Tashiro et al., 2009). The IC_{50} values of outliers were excluded in the calculation of the mean and standard deviation values for IC_{50}. A one-way analysis of variance was performed using Microsoft Excel 2010 software to compare the IC_{50} values among type/subtypes and NAIs. Statistical significance was set at an α value of 0.05.

2.6. NA gene sequencing

Viral RNA extraction, PCR, and sequencing of the full NA gene were performed as previously described (Dapat et al., 2009). Multiple sequence alignment was performed using the BioEdit software (http://www.mbio.ncsu.edu/BioEdit) with NA sequences of at least five drug-sensitive viruses in the same season to generate a consensus sequence. The consensus sequence of drug-sensitive viruses

Table 1
NAI data analysis of IC_{50} values (nM) grouped according to season and virus type/subtype.

Virus	Season	*N*	Oseltamivir			Zanamivir			Peramivir			Laninamivir			*P* value[c]
			Range	Mean ± SD[a]	Cutoff[b]	Range	Mean ± SD[a]	Cutoff[b]	Range	Mean ± SD[a]	Cutoff[b]	Range	Mean ± SD[a]	Cutoff[b]	
A(H1N1)pdm09	2009–2010	57	1.48–10.91	1.88 ± 0.25	3.43	0.49–1.92	0.69 ± 0.09	1.11	0.04–0.22	0.07 ± 0.01	0.12	0.16–0.49	0.24 ± 0.05	0.46	<0.0001
A(H1N1)pdm09	2010–2011	60	0.72–443.95	1.37 ± 0.31	2.61	0.30–1.79	0.63 ± 0.14	1.29	0.07–37.09	0.12 ± 0.03	0.28	0.20–0.66	0.27 ± 0.05	0.50	<0.0001
A(H3N2)	2010–2011	59	0.46–2.12	0.73 ± 0.20	1.69	0.41–2.66	0.63 ± 0.17	1.50	0.10–0.33	0.17 ± 0.04	0.37	0.30–1.09	0.65 ± 0.15	1.20	<0.0001
B	2010–2011	18	16.97–59.12	27.09 ± 11.00	70.08	29.33–92.30	53.74 ± 17.25	128.21	1.22–4.77	2.31 ± 0.88	4.48	4.69–15.23	8.44 ± 3.33	19.47	<0.0001
P value[3]				<0.0001			<0.0001			<0.0001			<0.0001		

[a] Mean IC_{50} value (nM) ± standard deviation (SD) was calculated from viruses with IC_{50} values below the cutoff value (IC_{50} value of outliers was excluded in the analysis).
[b] Cutoff value was determined based on the IC_{50} value of >75th percentile + 3 IQR.
[c] One-way analysis of variance ($\alpha = 0.05$) was performed to compare the IC_{50} values for each type/subtype and NAI.

for each type/subtype was compared with the sequences from isolates with reduced NAI susceptibility. Identified mutations were then compared to reported mutations that are known markers of resistance (Pozo et al., 2013; WHO_Global_Influenza_Surveillance_Network, 2011). All NA mutations are given in N2 subtype numbering (Colman et al., 1993). Sequences were submitted to GenBank with the following accession numbers, GenBank ID: CY066034 (A/Fukushima/09FY007/2009), CY066056 (A/Fukushima/09FY090/2009), CY066122 (A/Kyoto/09K266-2/2009), JN790409 (A/Hyogo/10K291/2011), CY110778 (A/Kyoto/10K070/2011), CY110779 (A/Kyoto/10K073/2011), JN790415 (A/Kyoto/10K124/2011), CY110782 (A/Nagasaki/10N017/2011), CY110777 (A/Niigata/10F010/2011), CY110780 (A/Kyoto/10K104-2/2011), and CY110781 (A/Kyoto/10K129-2/2011).

2.7. 3D modeling

Identified amino acid substitutions were mapped to NA. Crystal structure of NA from A/California/4/2009 virus (subtype H1N1pdm09) (PDB ID: 3TI6) (Vavricka et al., 2011) was downloaded from Protein Data Bank (RCSB PDB, http://www.pdb.org) (Berman et al., 2000). Molecular model was generated using the PyMol software v1.3 (http://www.pymol.org).

3. Results

3.1. Screening for NAI resistance

In the 2009–2010 season, 601 A(H1N1)pdm09 isolates were obtained from 733 first visit samples (before drug treatment) and 10 A(H1N1)pdm09 isolates were obtained from 42 second visit samples (after drug treatment) (Dapat et al., 2012). Of these, 57 representative first visit A(H1N1)pdm09 isolates that were selected randomly from each prefecture were tested for susceptibility to the four NAIs. In addition, all 10 A(H1N1)pdm09 isolates that were obtained from patients after antiviral treatment were assayed. In the 2010–2011 season, 414 A(H1N1)pdm09, 525 A(H3N2), and 33 type B viruses were obtained from 1278 first visit samples and nine A(H1N1)pdm09, four A(H3N2), and 1 type B isolates were obtained from 67 second visit samples. Of these, 60 A(H1N1)pdm09, 59 A(H3N2), and 18 type B isolates from first visit patients were tested for NAI assay. In addition, all isolates (eight H1N1pdm09 and 1 type B) from second visit patients were also tested by IC_{50} method.

Comparison of the IC_{50} values showed significant differences ($P < 0.0001$) among the four NAIs for each type/subtype (Table 1). The mean IC_{50} values among A(H1N1)pdm09 isolates were low to all four NAIs. Similarly, the mean IC_{50} values of A(H3N2) isolates were also low to oseltamivir (0.73 nM), zanamivir (0.63 nM), peramivir (0.17 nM), and laninamivir (0.65 nM). Among type A viruses, the A(H1N1)pdm09 strains had lower peramivir and laninamivir IC_{50} values than A(H3N2) viruses ($P < 0.0001$). On the other hand, A(H3N2) viruses had lower IC_{50}s to oseltamivir (0.73 nM) than A(H1N1)pdm09 strains (1.37 nM) ($P < 0.0001$). Influenza A(H1N1)pdm09 and A(H3N2) viruses exhibited similar IC_{50}s for zanamivir. The IC_{50} values of type B viruses were significantly higher ($P < 0.0001$) against oseltamivir and zanamivir with ranges from 1.97 to 59.12 nM and 29.33 to 92.3 nM, than peramivir (IC_{50} ranges from 1.22 to 4.77 nM) and laninamivir (IC_{50} ranges from 4.69 to 15.23 nM), respectively.

3.2. Characterization of outlier influenza viruses collected before antiviral treatment

Among the A(H1N1)pdm09 viruses in the 2009–2010 season that were collected from patients on their first visit, two mild

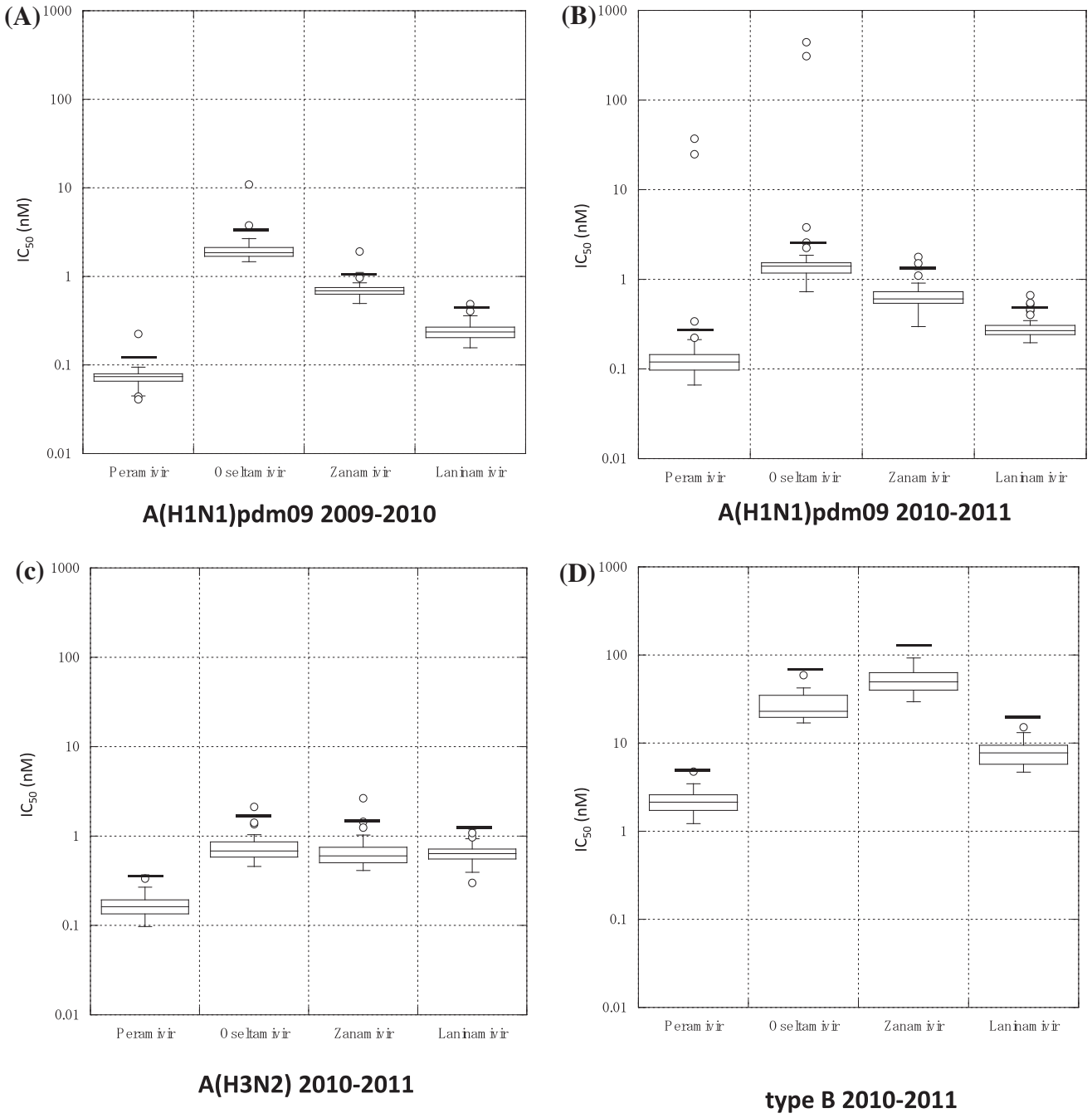

Fig. 1. Box-and-whisker plots showing IC_{50} values (in nM) of the four NAIs (peramivir, oseltamivir, zanamivir, and laninamivir) for influenza A(H1N1)pdm09 viruses in (A) 2009–2010 and (B) 2010–2011, (C) A(H3N2) and (D) type B viruses. Isolates (open circles) that are above the statistical cutoff (black horizontal line) of IC_{50} value with more than three times the interquartile range (IQR) from the 75th percentile were considered outliers.

outliers were detected out of 57 samples tested (Fig. 1A). One outlier (A/Fukushima/09FY007/2009) had elevated IC_{50} values for all four NAIs (Table 2). NA sequencing of both primary clinical sample and virus isolate showed a single mutation (I222T). Another mild outlier (A/Fukushima/09FY090/2009) had a 2-fold increase in oseltamivir IC_{50} value (3.78 nM) and harbored the M241I mutation. No H274Y viruses were detected among the first visit samples in the 2009–2010 season.

In the 2010–2011 influenza season, four outliers were identified among the 60 A(H1N1)pdm09 samples that were collected prior to antiviral treatment (Fig. 1B). Two extreme outliers (A/Hyogo/10K291/2011 and A/Kyoto/10K124/2011) had >200-fold increase in IC_{50} values against oseltamivir and peramivir (Table 2). Sequence analysis of the NA gene showed H274Y mutation in NA, which confers resistance to oseltamivir (Hauge et al., 2009; Hurt et al., 2011a; Ujike, et al., 2011). Three additional substitutions in NA (N49S, V240I, and N373K) of A/Hyogo/10K291/2011 and A/Kyoto/10K124/2011 viruses were also detected among other 2010–2011 isolates when compared with the NA sequence of drug-sensitive virus (A/Hyogo/09K305/2009; CY066126) from the previous season (2009–2010) (Dapat et al., 2012). The A/Hyogo/10K291/2011 isolate was collected from a 7-year old female patient, whose fever resolved (<37.5 °C) within 24 h after zanamivir treatment. The A/Kyoto/10K124/2011 isolate was obtained from

Table 2
Characterization of outlier influenza viruses that were collected before antiviral treatment.

Season	Type (subtype)	Virus	IC_{50} (nM) (fold change)[a]								NA mutation (s)[b] in primary samples	NA mutation (s)[b] in virus isolates
			Oseltamivir		Zanamivir		Peramivir		Laninamivir			
2009–2010	A(H1N1) pdm09	A/Fukushima/09FY007/2009	**10.91**	(5.8)	**1.92**	(2.8)	**0.22**	(3.2)	**0.49**	(2.1)	I222T	I222T
		A/Fukushima/09FY090/2009	**3.78**	(2.0)	0.49	(0.7)	0.09	(1.2)	0.20	(0.8)	M241I	M241I
2010–2011	A(H1N1) pdm09	A/Hyogo/10K291/2011	***443.95***	(323.5)	1.11	(1.8)	***37.09***	(307.8)	**0.54**	(2.0)	H274Y,E51G	H274Y,E51G
		A/Kyoto/10K070/2011	2.57	(1.9)	**1.79**	(2.8)	0.22	(1.8)	0.46	(1.7)	P120L,N221D	P120L,N221D
		A/Kyoto/10K073/2011	**3.80**	(2.8)	**1.51**	(2.4)	**0.34**	(2.8)	**0.66**	(2.4)	V263I,F322L,I362V N401K,S443I	V263I,F322L,I362V, N401K,S443I
		A/Kyoto/10K124/2011	***309.79***	(225.8)	0.85	(1.4)	***24.85***	(206.3)	0.40	(1.5)	H274Y,T384A	H274Y,S62N, V176G,R419T
2010–2011	A(H3N2)	A/Nagasaki/10N017/2011	**2.12**	(2.9)	0.77	(1.2)	0.14	(0.9)	0.30	(0.5)	None	D151G
		A/Niigata/10F010/2011	0.68	(0.9)	**2.66**	(4.2)	0.20	(1.2)	1.09	(1.7)	None	T148(T/I)[c]

[a] Fold change was calculated as a ratio between the sample's IC_{50} value and the mean IC_{50} value. Boldface indicates IC_{50} value which is more than three times the interquartile range (IQR) from the 75th percentile (mild outlier) and italicized boldface indicates IC_{50} value which is more than three times the IQR from the 75th percentile and at least 10-fold higher than the mean (extreme outlier).
[b] Amino acid position of NA mutation was based on N2 numbering.
[c] Mixed population based on sequencing result.

Table 3
Characterization of influenza viruses collected before and after antiviral treatment in the 2009–2010 season.

Patient	Age (year)	Treatment		Virus	Type(subtype)	Collection interval (days)	IC_{50} (nM)(fold change)[a]								NA mutation(s)[b]
							Oseltamivir		Zanamivir		Peramivir		Laninamivir		
A	9	None	Pre	A/Kyoto/09K167/2009	A(H1N1)pdm09		1.67		0.66		0.07		0.24		–
			Post	A/Kyoto/09K167-2/2009		3	1.58	(0.94)	0.59	(0.90)	0.06	(0.86)	0.20	(0.84)	–
B	7	None	Pre	A/Kyoto/09K172/2009	A(H1N1)pdm09		1.67		0.69		0.07		0.21		–
			Post	A/Kyoto/09K172-2/2009		3	1.67	(1.00)	0.69	(0.99)	0.07	(0.96)	0.23	(1.07)	–
C	5	Oseltamivir	Pre	A/Kyoto/09K204/2009	A(H1N1)pdm09		1.66		0.73		0.07		0.36		–
			Post	A/Kyoto/09K204-2/2009		3	1.58	(0.96)	0.72	(0.98)	0.07	(1.06)	0.31	(0.87)	–
D	5	Oseltamivir	Pre	A/Kyoto/09K266/2009	A(H1N1)pdm09		1.74		0.62		0.08		0.23		No mutation
			Post	A/Kyoto/09K266-2/2009		3	***440.13***	(252.79)	0.85	(1.36)	***30.95***	(389.22)	0.46	(1.98)	H274Y
E	7	None	Pre	A/Kyoto/09K286/2009	A(H1N1)pdm09		1.68		0.77		0.07		0.24		–
			Post	A/Kyoto/09K286-2/2009		4	1.70	(1.01)	0.69	(0.90)	0.08	(1.08)	0.22	(0.93)	–
F	5	None	Pre	A/Kyoto/09K295(3)/2009	A(H1N1)pdm09		1.58		0.68		0.07		0.28		–
			Post	A/Kyoto/09K295(3)-2/2009		5	1.92	(1.22)	0.82	(1.22)	0.08	(1.26)	0.24	(0.83)	–
G	8	Oseltamivir	Pre	A/Kyoto/09K318/2009	A(H1N1)pdm09		1.48		0.55		0.06		0.20		–
			Post	A/Kyoto/09K318-2/2009		4	2.30	(1.56)	1.03	(1.87)	0.07	(1.20)	0.23	(1.12)	–
H	8	Oseltamivir	Pre	A/Kyoto/09K320/2009	A(H1N1)pdm09		1.93		0.68		0.07		0.22		–
			Post	A/Kyoto/09K320-2/2009		4	1.64	(0.85)	0.53	(0.78)	0.06	(0.79)	0.21	(0.94)	–
I	5	Oseltamivir	Pre	A/Kyoto/09K327/2009	A(H1N1)pdm09		1.65		0.57		0.06		0.25		–
			Post	A/Kyoto/09K327-2/2009		4	1.70	(1.03)	0.69	(1.20)	0.07	(1.14)	0.23	(0.90)	–
J	4	Oseltamivir	Pre	A/Kyoto/09K328/2009	A(H1N1)pdm09		1.95		0.76		0.08		0.21		–
			Post	A/Kyoto/09K328-2/2009		4	1.86	(0.96)	0.71	(0.93)	0.07	(0.97)	0.19	(0.90)	–

– indicates not determined.
[a] Fold change was calculated as a ratio between the sample's IC_{50} value and the mean IC_{50} value. Boldface indicates IC_{50} value which is more than three times the interquartile range (IQR) from the 75th percentile (mild outlier) and italicized boldface indicates IC_{50} value which is more than three times the IQR from the 75th percentile and at least 10-fold higher than the mean (extreme outlier).
[b] Amino acid position of NA mutation was based on N2 numbering. NA sequence was derived from virus isolate after three passages in MDCK cells.

a 5-year female patient, whose fever resolved within 48 h after oseltamivir therapy. No prolongation of fever of the two patients was noted by clinicians. The respective schools of the two patients reported an influenza outbreak during the sample collection period. The patients' families reported influenza infection after the two patients got infected. However, samples and NAI treatment history from the families and schoolmates were not available for analysis in this study. One mild outlier (A/Kyoto/10K073/2011) had elevated IC_{50} values for all four NAIs. Sequencing analysis of both the primary sample and virus isolate showed five amino acid mutations in NA. Another mild outlier (A/Kyoto/10K070/2011) had a >2-fold increase in IC_{50} for zanamivir and possessed the P120L and N221D mutations.

Among the A(H3N2) isolates that were collected before antiviral treatment, two mild outliers were identified out of 59 samples tested (Fig. 1C). The A/Nagasaki/10N017/2011 isolate with elevated IC_{50} value for oseltamivir (2.12 nM) harbored the D151G mutation (Table 2). The A/Nagasaki/10N017/2011 isolate was passaged in MDCK cells three times. However, the D151G mutation was not detected in the primary clinical sample. The A/Niigata/10F010/2011 isolate exhibited a 4-fold increase in IC_{50} for zanamivir (2.66 nM) and possessed the T148T/I (mixture of T and I) mutation in NA. Sequencing of the primary clinical sample likewise showed only wild-type sequence.

For influenza B viruses, statistical analysis of the IC_{50} values for all four NAIs showed no outliers (Fig. 1D).

3.3. Characterization of viruses collected before and after antiviral treatment

Among the paired A(H1N1)pdm09 samples collected during the 2009–2010 and 2010–2011 seasons, three outliers (3/11; 27.3%)

were identified in the oseltamivir-treatment group (Table 3). No outliers were identified in the laninamivir-treatment group and non-treatment group. Of the three outliers among A(H1N1)pdm09 viruses, one extreme outlier was identified from the 2009–2010 season ($n = 6$ pairs in oseltamivir treatment group) and one extreme outlier and one mild outlier were detected from the 2010–2011 season ($n = 5$ pairs in oseltamivir treatment group). The A/Kyoto/09K266-2/2009 isolate was collected from patient D, a 6-year old female, on the third day of oseltamivir treatment and exhibited >200-fold increase in IC_{50} values against oseltamivir and peramivir (Table 3). NA sequencing result obtained from virus isolate showed a single H274Y mutation (Table 3). The A/Kyoto/10K104-2/2011 sample was collected from patient C, a 4-year old male, on the third day of oseltamivir treatment, which exhibited high IC_{50} values for both oseltamivir and peramivir and possessed the H274Y mutation (Table 4). One mild outlier (A/Kyoto/10K129-2/2011) that was collected from patient F, a 2-year old female, after 2 days of oseltamivir treatment showed only a slight increase in IC_{50} value for oseltamivir (2.8 nM) (Table 4). Sequence analysis revealed a mixed population of wild-type and H274Y mutants. Patient C showed fever resolution within 24 h but both patients D and F had prolonged fever for 3 days after oseltamivir treatment.

One paired influenza B sample that was collected from a patient who underwent zanamivir treatment showed no increase in IC_{50} values (Table 4).

4. Discussion

The antiviral drug susceptibility profile of pandemic and seasonal influenza viruses to oseltamivir, zanamivir, peramivir, and laninamivir was determined in this study. The prevalence of oseltamivir-resistant A(H1N1)pdm09 viruses with H274Y mutation collected from patients before antiviral drug treatment increased from 0% (0/54) in the 2009–2010 season to 3% (2/60) in the 2010–2011 season. The nationwide surveillance data of oseltamivir resistance in Japan also reported an increasing trend of detection of H274Y mutants from 1.0% in the 2009–2010 season to 2.0% in the following season (Infectious_Disease_Surveillance_Center, 2012). Despite the widespread use of NAIs in Japan, the prevalence of oseltamivir resistance in the community remained low (Sugaya, 2011). This low prevalence of oseltamivir resistance is comparable with surveillance data in other countries (Longtin et al., 2011; Okomo-Adhiambo et al., 2010b; Puzelli et al., 2011; Ujike et al., 2011). The H274Y mutation also showed reduced susceptibility to peramivir but not to zanamivir and laninamivir.

We detected two cases of A(H1N1)pdm09 virus infection with H274Y mutation from first visit patients before antiviral treatment, which may suggest that the H274Y mutation occurred spontaneously or the H274Y virus was acquired through community transmission. On one hand, cases of naturally occurring A(H1N1)pdm09 viruses with H274Y mutation had been reported in Japan, Hong Kong, and Vietnam (Chen et al., 2009; Le et al., 2010; Ujike et al., 2011) and on the other hand, cases of person-to-person transmission of oseltamivir-resistant A(H1N1)pdm09 viruses were reported in Australia and in the United Kingdom (Hurt et al., 2011b, 2012; Moore et al., 2011). In this study, one patient received oseltamivir and the other received zanamivir treatment. Since the H274Y virus is sensitive to zanamivir, this may suggest a possibility of community transmission of H274Y viruses. However, we could not confirm the source of drug-resistant strain in our study. It was reported that the three permissive mutations (V240I, N373K, and N390S) in NA may have contributed to the increase in viral fitness of H274Y viruses that caused widespread community transmission in Australia (Hurt et al., 2012). NA sequence analysis showed that the Japanese H274Y viruses possessed only two of the three re-

Table 4
Characterization of influenza viruses collected before and after antiviral treatment in the 2010–2011 season.

Patient	Age (year)	Treatment	Virus		Type(subtype)	Collection interval (days)	IC_{50}(nM)(fold change)[a]						NA mutation(s)[b]
							Oseltamivir		Zanamivir		Peramivir		Laninamivir		
A	12	Laninamivir	Pre	A/Kyoto/10K072/2011	A(H1N1)pdm09		1.68		0.81		0.15		0.27		–
			Post	A/Kyoto/10K072-2/2011		3	1.45	(0.86)	0.75	(0.92)	0.19	(1.23)	0.31	(1.16)	–
B	1	Oseltamivir	Pre	A/Kyoto/10K099/2011	A(H1N1)pdm09		1.16		0.50		0.11		0.22		No mutation
			Post	A/Kyoto/10K099-2/2011		4	1.21	(1.04)	0.83	(1.68)	0.07	(0.63)	0.25	(1.16)	I264 V
C	4	Oseltamivir	Pre	A/Kyoto/10K104/2011	A(H1N1)pdm09		1.56		0.77		0.18		0.34		D416G
			Post	A/Kyoto/10K104-2/2011		3	***386.94***	(248.19)	0.97	(1.27)	***29.94***	(167.48)	0.56	(1.64)	H274Y
D	3	Oseltamivir	Pre	A/Kyoto/10K106/2011	A(H1N1)pdm09		1.53		0.71		0.17		0.27		–
			Post	A/Kyoto/10K106-2/2011		4	2.04	(1.33)	0.88	(1.23)	0.14	(0.86)	0.28	(1.04)	–
E	2	Oseltamivir	Pre	A/Kyoto/10K113/2011	A(H1N1)pdm09		1.52		0.91		0.09		0.26		–
			Post	A/Kyoto/10K113-2/2011		3	1.38	(0.91)	1.09	(1.20)	0.09	(1.03)	0.28	(1.11)	–
F	1	Oseltamivir	Pre	A/Kyoto/10K129/2011	A(H1N1)pdm09		1.65		0.66		0.08		0.29		No mutation
			Post	A/Kyoto/10K129-2/2011		2	**2.80**	(1.70)	0.58	(0.88)	0.14	(1.65)	0.24	(0.82)	274H/Y[3]
G	15	Laninamivir	Pre	A/Kyoto/10K146/2011	A(H1N1)pdm09		1.27		0.91		0.10		0.25		–
			Post	A/Kyoto/10K146-2/2011		3	1.41	(1.11)	0.92	(1.01)	0.09	(0.95)	0.27	(1.05)	–
H	9	Laninamivir	Pre	A/Kyoto/10K179/2011	A(H1N1)pdm09		1.19		0.56		0.08		0.30		–
			Post	A/Kyoto/10K179-2/2011		4	1.24	(1.05)	0.52	(0.93)	0.08	(1.00)	0.28	(0.93)	–
I	8	Zanamivir	Pre	B/Kyoto/10K436/2011	B		39.12		37.27		3.44		7.30		–
			Post	B/Kyoto/10K436-2/2011		3	31.34	(0.80)	31.69	(0.85)	2.45	(0.71)	7.00	(0.96)	–

– indicates not determined.

[a] Fold change was calculated as a ratio between the sample's IC_{50} value and the mean IC_{50} value. Boldface indicates IC_{50} value which is more than three times the interquartile range (IQR) from the 75th percentile (mild outlier) and italicized boldface indicates IC_{50} value which is more than three times the IQR from the 75th percentile and at least 10-fold higher than the mean (extreme outlier).

[b] Amino acid position of NA mutation was based on N2 numbering. NA sequence was derived from virus isolate after three passages in MDCK cells.

[3] Mixed population based on sequencing result.

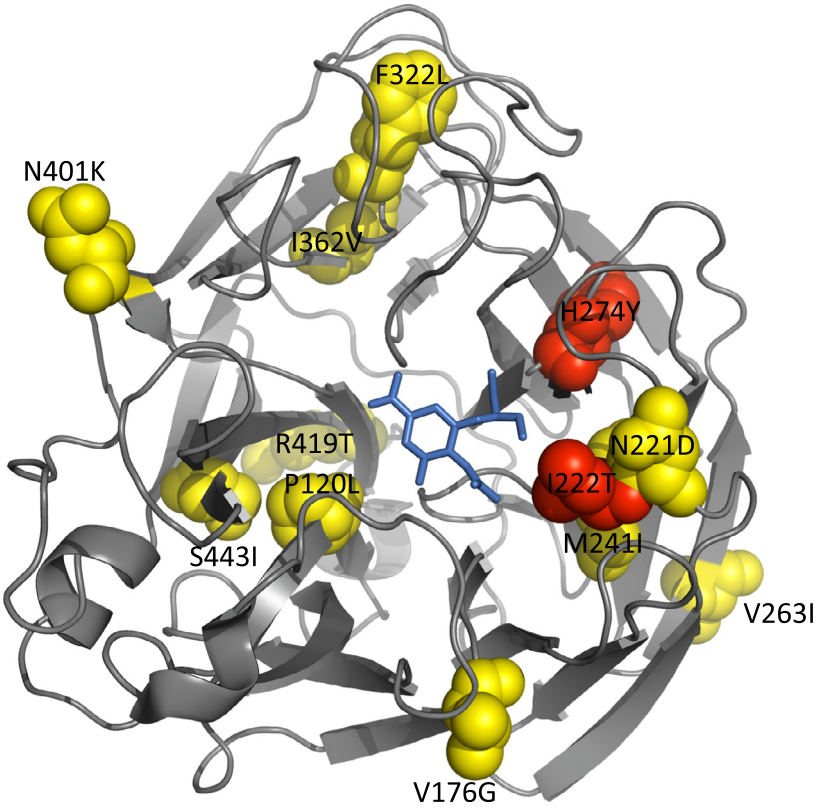

Fig. 2. Amino acid substitutions in the NA of outlier influenza A(H1N1)pdm09 viruses that were collected in Japan, 2009–2010 and 2010–2011. Three-dimensional structure of monomeric NA with bound oseltamivir (blue) (subtype H1N1pdm09, PDB ID: 3TI6) (Vavricka et al., 2011) was downloaded from the Protein Data Bank (RCSB PDB, http://www.pdb.org) (Berman et al., 2000). Mutations localized in the framework region are colored red. Mutations located outside the catalytic and framework residues are colored yellow. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

ported permissive mutations (V240I and N373K) (Dapat et al., 2012). This may explain the low detection rate of H274Y viruses in Japan. We have to continue close monitoring of H274Y mutation among A(H1N1)pdm09 viruses as well as the permissive mutations that would indicate community circulation of resistant strains.

We detected three (27.3%) A(H1N1)pdm09 isolates with H274Y mutation from samples that were collected during oseltamivir treatment, which suggests that the drug was responsible for the emergence of mutant viruses. Two A(H1N1)pdm09 samples, which were obtained 3 days after oseltamivir treatment showed elevated IC_{50} values for oseltamivir and peramivir. The detection of H274Y mutants from samples that were collected 3 days after drug therapy was also observed in previous reports (Sy et al., 2010; Ujike et al., 2011). Rapid emergence of H274Y mutants was detected in one A(H1N1)pdm09 sample that was collected 48 h after oseltamivir treatment, however, its oseltamivir IC_{50} value was lower and sequencing results showed a mixed population of wild-type and mutant variants. This mixed population was detected during the initial screening by cycling-probe real-time PCR assay to identify the single nucleotide polymorphisms (SNPs) for genotyping oseltamivir-sensitive (H274) and oseltamivir-resistant (Y274) viruses (Dapat et al., 2012). An earlier study showed that 24% and 52% of the virus sequences analyzed by pyrosequencing had the H274Y mutation from samples that were collected 38 and 45 h, respectively after oseltamivir therapy (Inoue et al., 2010).

Several mutations in the NA gene were identified among mild outlier viruses. One influenza A(H1N1)pdm09 isolate with an I222T mutation was identified as a mild outlier for all four NAIs. The sample was collected before drug treatment suggesting that the mutation may have occurred spontaneously. The I222T mutation was also detected in the primary clinical sample. A single I222K mutation was detected from an A(H1N1)pdm09 isolate, causing a 14-fold increase in IC_{50} for oseltamivir (Okomo-Adhiambo et al., 2010b). While dual mutations, H274Y and I222R were detected from two immunocompromised patients after oseltamivir or oseltamivir and zanamivir treatment, causing elevated IC_{50}s for oseltamivir and peramivir and moderate resistance to zanamivir (Nguyen et al., 2010a; van der Vries et al., 2010). The amino acid substitution at position 222 is a framework site and may explain the reduction of susceptibility to all four NAIs (Fig. 2) (Colman et al., 1993). The different mutations at position 222 in NA suggest its importance as molecular markers for drug resistance (van der Vries et al., 2011). Other NA mutations, such as M241I (mild outlier for oseltamivir), and P120L and N221D (mild outlier for zanamivir) were detected from two A(H1N1)pdm09 samples, which were collected prior to drug treatment in this study. These mutations are novel and are located outside the catalytic and framework residues (Fig. 2) (Colman et al., 1993). These mutations caused mild increase in IC_{50} values; however, the molecular significance is currently unknown and may have no clinical impact. Future study will be investigated on the effect of these novel mutations in the presence of well-characterized drug resistance marker such as H274Y.

Two NA mutations in A (H3N2) viruses were detected during virus expansion in MDCK cells. The D151G mutation was identified in one isolate, which is a mild outlier to oseltamivir and the T148I mutation was found in another isolate, which is a mild outlier to zanamivir. Direct sequencing of the primary clinical samples showed only wild-type sequences suggesting that these mutations arise during virus propagation in MDCK cells (Lin et al., 2010; Okomo-Adhiambo, et al., 2010a). A possible explanation for the emer-

gence of mutant viruses is that the isolates were expanded in MDCK cells three times to achieve high titers for NAI assay. Isolates should be propagated to a maximum of two passages in order to prevent the emergence of mutant viruses during cell culture (Nguyen et al., 2010b). Limiting the passage number may also minimize compensatory mutations in hemagglutinin (HA) to balance the NA enzyme activity, which may affect the results of NAI assay (Okomo-Adhiambo et al., 2013). Thus, careful consideration must be taken when assessing outliers such that the emergence of the mutant viruses does not occur during virus propagation *in vitro* and it is necessary to check the sequence of the primary samples whether the mutations exist or not (Okomo-Adhiambo et al., 2010a,b).

To summarize, the prevalence of NAI resistance among influenza A(H1N1)pdm09 viruses was low but increasing in Japan in the 2009–2010 and 2010–2011 influenza seasons. No NAI resistance was found among influenza A(H3N2) and type B isolates in this study. Currently, it is difficult to compare the IC_{50} values with previously published results due to differences in the assay and analysis employed. Thus, the Antiviral Working Group of the WHO Global Influenza Surveillance and Response System (GISRS) has defined categories of reporting susceptibility of influenza virus to NAIs by comparing the fold-change of IC_{50} values to that of reference susceptible viruses (Pozo et al., 2013; World_Health_Organization, 2012). In our future study, we will adopt this guideline for categorizing NAI susceptibilities.

Acknowledgments

This study was funded by the Ministry of Health, Labor and Welfare of Japan. We would like to thank Akemi Watanabe and Kae Horie of the Division of International Health (Public Health), Graduate School of Medical and Dental Sciences, Niigata University, Niigata, Japan for their technical support. We would like to thank Emi Takashita of the National Institute of Infectious Diseases (NIID), Tokyo, Japan and Ryu Yoshida of Shionogi & Co., Ltd. Osaka, Japan for their technical advice on NAI assay. We would like to thank the members of the Japanese Influenza Collaborative Study Group: Shinji Kimura (Hokkaido); Takashi Kawashima (Gunma); Isamu Sato (Niigata); Taku Iizuka (Fukushima); Shigeyoshi Hibi, Satoshi Ikushima, Fumihiro Fujiwara, Kentaro Tsunamoto (Kyoto); Takao Yoshihara (Osaka); Tetsuo Hashida (Hyogo); and Hironori Masaki, Yutaka Shirahige, Hidefumi Ishikawa, Satoshi Degawa, Norichika Aso, Reiki Kuroki, Kazuhiko Hoshino, Yasuhiko Ono (Nagasaki).

References

Baranovich, T., Saito, R., Suzuki, Y., Zaraket, H., Dapat, C., Caperig Dapat, I., Oguma, T., Shabana, I.I., Saito, T., Suzuki, H.J. I. C. S. Group, 2010. Emergence of H274Y oseltamivir-resistant A(H1N1) influenza viruses in Japan during the 2008–2009 season. J. Clin. Virol. 47, 23–28.

Berman, H.M., Westbrook, J., Feng, Z., Gilliland, G., Bhat, T.N., Weissig, H., Shindyalov, I.N., Bourne, P.E., 2000. The protein data bank. Nucleic Acids Res. 28, 235–242.

Besselaar, T., Naidoo, D., Buys, A., Gregory, V., McAnerney, J., Manamela, J., Blumberg, L., Schoub, B., 2008. Widespread oseltamivir resistance in influenza A viruses (H1N1), South Africa. Emerg. Infect. Dis. 14, 1809–1810.

Chen, H., Cheung, C.L., Tai, H., Zhao, P., Chan, J.F., Cheng, V.C., Chan, K.H., Yuen, K.Y., 2009. Oseltamivir-resistant influenza A pandemic (H1N1) 2009 virus, Hong Kong, China. Emerg. Infect. Dis. 15, 1970–1972.

Colman, P.M., 1994. Influenza virus neuraminidase: structure, antibodies, and inhibitors. Protein Sci. 3, 1687–1696.

Colman, P.M., Hoyne, P.A., Lawrence, M.C., 1993. Sequence and structure alignment of paramyxovirus hemagglutinin-neuraminidase with influenza virus neuraminidase. J. Virol. 67, 2972–2980.

Dapat, C., Saito, R., Kyaw, Y., Naito, M., Hasegawa, G., Suzuki, Y., Dapat, I., Zaraket, H., Cho, T., Li, D., Oguma, T., Baranovich, T., Suzuki, H., 2009. Epidemiology of human influenza A and B viruses in Myanmar from 2005 to 2007. Intervirology 52, 310–320.

Dapat, I.C., Dapat, C., Baranovich, T., Suzuki, Y., Kondo, H., Shobugawa, Y., Saito, R., Suzuki, H.J.I.C.S. Group, 2012. Genetic characterization of human influenza viruses in the pandemic (2009–2010) and post-pandemic (2010–2011) periods in Japan. PLoS One 7, e36455.

Dharan, N., Gubareva, L., Meyer, J., Okomo-Adhiambo, M., McClinton, R., Marshall, S., St George, K., Epperson, S., Brammer, L., Klimov, A., Bresee, J., Fry, A., 2009. Infections with oseltamivir-resistant influenza A(H1N1) virus in the United States. JAMA 301, 1034–1041.

Gubareva, L.V., 2004. Molecular mechanisms of influenza virus resistance to neuraminidase inhibitors. Virus Res. 103, 199–203.

Hauge, S., Dudman, S., Borgen, K., Lackenby, A., Hungnes, O., 2009. Oseltamivir-resistant influenza viruses A (H1N1), Norway, 2007–08. Emerg. Infect. Dis. 15, 155–162.

Hurt, A., Barr, I., Hartel, G., Hampson, A., 2004. Susceptibility of human influenza viruses from Australasia and South East Asia to the neuraminidase inhibitors zanamivir and oseltamivir. Antiviral Res. 62, 37–45.

Hurt, A., Ernest, J., Deng, Y., Iannello, P., Besselaar, T., Birch, C., Buchy, P., Chittaganpitch, M., Chiu, S., Dwyer, D., Guigon, A., Harrower, B., Kei, I., Kok, T., Lin, C., McPhie, K., Mohd, A., Olveda, R., Panayotou, T., Rawlinson, W., Scott, L., Smith, D., D'Souza, H., Komadina, N., Shaw, R., Kelso, A., Barr, I., 2009. Emergence and spread of oseltamivir-resistant A(H1N1) influenza viruses in Oceania, South East Asia and South Africa. Antiviral Res. 83, 90–93.

Hurt, A.C., Deng, Y.M., Ernest, J., Caldwell, N., Leang, L., Iannello, P., Komadina, N., Shaw, R., Smith, D., Dwyer, D.E., Tramontana, A.R., Lin, R.T., Freeman, K., Kelso, A., Barr, I.G., 2011a. Oseltamivir-resistant influenza viruses circulating during the first year of the influenza A(H1N1) 2009 pandemic in the Asia-Pacific region, March 2009 to March 2010. Euro. Surveill. 16.

Hurt, A.C., Hardie, K., Wilson, N.J., Deng, Y.M., Osbourn, M., Gehrig, N., Kelso, A., 2011b. Community transmission of oseltamivir-resistant A(H1N1)pdm09 influenza. N. Engl. J. Med. 365, 2541–2542.

Hurt, A.C., Lee, R.T., Leang, S.K., Cui, L., Deng, Y.M., Phuah, S.P., Caldwell, N., Freeman, K., Komadina, N., Smith, D., Speers, D., Kelso, A., Lin, R.T., Maurer-Stroh, S., Barr, I.G., 2011c. Increased detection in Australia and Singapore of a novel influenza A(H1N1)2009 variant with reduced oseltamivir and zanamivir sensitivity due to a S247N neuraminidase mutation. Euro. Surveill. 16.

Hurt, A.C., Hardie, K., Wilson, N.J., Deng, Y.M., Osbourn, M., Leang, S.K., Lee, R.T., Iannello, P., Gehrig, N., Shaw, R., Wark, P., Caldwell, N., Givney, R.C., Xue, L., Maurer-Stroh, S., Dwyer, D.E., Wang, B., Smith, D.W., Levy, A., Booy, R., Dixit, R., Merritt, T., Kelso, A., Dalton, C., Durrheim, D., Barr, I.G., 2012. Characteristics of a widespread community cluster of H275Y oseltamivir-resistant A(H1N1)pdm09 influenza in Australia. J. Infect. Dis. 206, 148–157.

Infectious_Disease_Surveillance_Center (2012). Flash Report on Influenza Virus in Japan: City.

Inoue, M., Barkham, T., Leo, Y.S., Chan, K.P., Chow, A., Wong, C.W., Lee, Tze Chuen, Maurer-Stroh, R., Lin, S., Lin, C., 2010. Emergence of oseltamivir-resistant pandemic (H1N1) 2009 virus within 48 hours. Emerg. Infect. Dis. 16, 1633–1636.

Kohno, S., Kida, H., Mizuguchi, M., Shimada, J.S.-C. S. Group, 2010. Efficacy and safety of intravenous peramivir for treatment of seasonal influenza virus infection. Antimicrob. Agents Chemother. 54, 4568–4574.

Le, Q.M., Wertheim, H.F., Tran, N.D., van Doorn, H.R., Nguyen, T.H., Horby, P.V.H.N.I. Team, 2010. A community cluster of oseltamivir-resistant cases of 2009 H1N1 influenza. N. Engl. J. Med. 362, 86–87.

Lin, Y.P., Gregory, V., Collins, P., Kloess, J., Wharton, S., Cattle, N., Lackenby, A., Daniels, R., Hay, A., 2010. Neuraminidase receptor binding variants of human influenza A(H3N2) viruses resulting from substitution of aspartic acid 151 in the catalytic site: a role in virus attachment? J. Virol. 84, 6769–6781.

Longtin, J., Patel, S., Eshaghi, A., Lombos, E., Higgins, R., Alexander, D., Olsha, R., Doyle, J., Tran, D., Sarabia, A., Lee, C., Bastien, N., Li, Y., Low, D., Boivin, G., Gubbay, J., 2011. Neuraminidase-inhibitor resistance testing for pandemic influenza A (H1N1) 2009 in Ontario, Canada. J. Clin. Virol. 50, 257–261.

McKimm-Breschkin, J., Trivedi, T., Hampson, A., Hay, A., Klimov, A., Tashiro, M., Hayden, F., Zambon, M., 2003. Neuraminidase sequence analysis and susceptibilities of influenza virus clinical isolates to zanamivir and oseltamivir. Antimicrob. Agents Chemother. 47, 2264–2272.

Monto, A., McKimm-Breschkin, J., Macken, C., Hampson, A., Hay, A., Klimov, A., Tashiro, M., Webster, R., Aymard, M., Hayden, F., Zambon, M., 2006. Detection of influenza viruses resistant to neuraminidase inhibitors in global surveillance during the first 3 years of their use. Antimicrob. Agents Chemother. 50, 2395–2402.

Moore, C., Galiano, M., Lackenby, A., Abdelrahman, T., Barnes, R., Evans, M.R., Fegan, C., Froude, S., Hastings, M., Knapper, S., Litt, E., Price, N., Salmon, R., Temple, M., Davies, E., 2011. Evidence of person-to-person transmission of oseltamivir-resistant pandemic influenza A(H1N1) 2009 virus in a hematology unit. J. Infect. Dis. 203, 18–24.

Moscona, A., 2005. Neuraminidase inhibitors for influenza. N. Engl. J. Med. 353, 1363–1373.

Mungall, B.A., Xu, X., Klimov, A., 2004. Surveillance of influenza isolates for susceptibility to neuraminidase inhibitors during the 2000–2002 influenza seasons. Virus Res. 103, 195–197.

Nguyen, H.T., Fry, A.M., Loveless, P.A., Klimov, A.I., Gubareva, L.V., 2010a. Recovery of a multidrug-resistant strain of pandemic influenza A 2009 (H1N1) virus carrying a dual H275Y/I223R mutation from a child after prolonged treatment with oseltamivir. Clin. Infect. Dis. 51, 983–984.

Nguyen, H.T., Sheu, T.G., Mishin, V.P., Klimov, A.I., Gubareva, L.V., 2010b. Assessment of pandemic and seasonal influenza A (H1N1) virus susceptibility to neuraminidase inhibitors in three enzyme activity inhibition assays. Antimicrob. Agents Chemother. 54, 3671–3677.

Okomo-Adhiambo, M., Nguyen, H., Sleeman, K., Sheu, T., Deyde, V., Garten, R., Xu, X., Shaw, M., Klimov, A., Gubareva, L., 2010a. Host cell selection of influenza neuraminidase variants: implications for drug resistance monitoring in A(H1N1) viruses. Antiviral Res. 85, 381–388.

Okomo-Adhiambo, M., Sleeman, K., Ballenger, K., Nguyen, H.T., Mishin, V.P., Sheu, T.G., Smagala, J., Li, Y., Klimov, A.I., Gubareva, L.V., 2010b. Neuraminidase inhibitor susceptibility testing in human influenza viruses: a laboratory surveillance perspective. Viruses 2, 2269–2289.

Okomo-Adhiambo, M., Sheu, T.G., Gubareva, L.V., 2013. Assays for monitoring susceptibility of influenza viruses to neuraminidase inhibitors. Influenza Other Respir. Viruses 7 (Suppl. 1), 44–49.

Pozo, F., Lina, B., Andrade, H.R., Enouf, V., Kossyvakis, A., Broberg, E., Daniels, R., Lackenby, A., Meijer, A., 2013. Guidance for clinical and public health laboratories testing for influenza virus antiviral drug susceptibility in Europe. J. Clin. Virol..

Puzelli, S., Facchini, M., Di Martino, A., Fabiani, C., Lackenby, A., Zambon, M., Donatelli, I., 2011. Evaluation of the antiviral drug susceptibility of influenza viruses in Italy from 2004/05 to 2009/10 epidemics and from the recent 2009 pandemic. Antiviral Res. 90, 205–212.

Sheu, T.G., Deyde, V.M., Okomo-Adhiambo, M., Garten, R.J., Xu, X., Bright, R.A., Butler, E.N., Wallis, T.R., Klimov, A.I., Gubareva, L.V., 2008. Surveillance for neuraminidase inhibitor resistance among human influenza A and B viruses circulating worldwide from 2004 to 2008. Antimicrob. Agents Chemother. 52, 3284–3292.

Sugaya, N., 2011. Widespread use of neuraminidase inhibitors in Japan. J. Infect. Chemother. 17, 595–601.

Sy, C.L., Lee, S.S., Liu, M.T., Tsai, H.C., Chen, Y.S., 2010. Rapid emergence of oseltamivir resistance. Emerg. Infect. Dis. 16, 723–725.

Tashiro, M., McKimm-Breschkin, J., Saito, T., Klimov, A., Macken, C., Zambon, M., Hayden, F., 2009. Surveillance for neuraminidase-inhibitor-resistant influenza viruses in Japan, 1996–2007. Antivir. Ther. 14, 751–761.

Ujike, M., Shimabukuro, K., Mochizuki, K., Obuchi, M., Kageyama, T., Shirakura, M., Kishida, N., Yamashita, K., Horikawa, H., Kato, Y., Fujita, N., Tashiro, M., Odagiri, T.Japan W.G.f.I.V.S.i, 2010. Oseltamivir-resistant influenza viruses A (H1N1) during 2007–2009 influenza seasons, Japan. Emerg. Infect. Dis. 16, 926–935.

Ujike, M., Ejima, M., Anraku, A., Shimabukuro, K., Obuchi, M., Kishida, N., Hong, X., Takashita, E., Fujisaki, S., Yamashita, K., Horikawa, H., Kato, Y., Oguchi, A., Fujita, N., Tashiro, M., Odagiri, T.Japan a.t.I.V.S.G.o, 2011. Monitoring and characterization of Oseltamivir-resistant pandemic (H1N1) 2009 virus, Japan, 2009–2010. Emerg. Infect. Dis. 17, 470–479.

van der Vries, E., Stelma, F.F., Boucher, C.A., 2010. Emergence of a multidrug-resistant pandemic influenza A (H1N1) virus. N. Engl. J. Med. 363, 1381–1382.

van der Vries, E., Veldhuis Kroeze, E.J., Stittelaar, K.J., Linster, M., Van der Linden, A., Schrauwen, E.J., Leijten, L.M., van Amerongen, G., Schutten, M., Kuiken, T., Osterhaus, A.D., Fouchier, R.A., Boucher, C.A., Herfst, S., 2011. Multidrug resistant 2009 A/H1N1 influenza clinical isolate with a neuraminidase I223R mutation retains its virulence and transmissibility in ferrets. PLoS Pathog. 7, e1002276.

Vavricka, C.J., Li, Q., Wu, Y., Qi, J., Wang, M., Liu, Y., Gao, F., Liu, J., Feng, E., He, J., Wang, J., Liu, H., Jiang, H., Gao, G.F., 2011. Structural and functional analysis of laninamivir and its octanoate prodrug reveals group specific mechanisms for influenza NA inhibition. PLoS Pathog. 7, e1002249.

WHO_Global_Influenza_Surveillance_Network (2011). Manual for the laboratory diagnosis and virological surveillance of influenza: City. World_Health_Organization (2012). Meetings of the WHO working group on surveillance of influenza antiviral susceptibility – Geneva, November 2011 and June 2012: City, pp. 369–374.

Yamashita, M., Tomozawa, T., Kakuta, M., Tokumitsu, A., Nasu, H., Kubo, S., 2009. CS-8958, a prodrug of the new neuraminidase inhibitor R-125489, shows long-acting anti-influenza virus activity. Antimicrob. Agents Chemother. 53, 186–192.