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Early findings of oseltamivir-resistant pandemic (H1N1) 2009 influenza A viruses in Taiwan

Ji-Rong Yang[a], Yuan-Pin Huang[a], Yu-Cheng Lin[a], Chun-Hui Su[a], Chuan-Yi Kuo[a], Li-Ching Hsu[a], Ho-Sheng Wu[a,b,*], Ming-Tsan Liu[a,**]

[a] National Influenza Center, Centers for Disease Control, Taipei, Taiwan, ROC
[b] School of Medical Laboratory Science and Biotechnology, Taipei Medical University, Taipei, Taiwan, ROC

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ABSTRACT

In this study, we investigated the frequency of oseltamivir resistance in pandemic (H1N1) 2009 influenza A viruses in Taiwan and characterized the resistant viruses. From May 2009 to January 2010, 1187 pandemic H1N1 virus-positive cases in Taiwan were tested for the H275Y substitution in the neuraminidase (NA) gene that confers resistance to oseltamivir. Among them, eight hospitalized cases were found to be infected with virus encoding the H275Y substitution in their original specimens collected after oseltamivir treatment. The epidemiologic investigation indicated that each of the cases occurred sporadically and there was no evidence of further transmission. We monitored the variation of amino acid residues at position 275 of the NA gene in a series of specimens taken at various time-points and observed that viruses encoding the H275Y substitution differ in their fitness in vivo and in MDCK cells. Phylogenetic analysis indicated that the hemagglutinin (HA) sequences of oseltamivir-resistant pandemic H1N1 viruses exhibited greater diversity than the NA sequences and progressive changes of the HA genes from clade A1 into A2 and from there into clade A3 were observed. The resistant viruses seemed to occur in combination with diverse HA genes and a dominant NA gene. Enzymatic analysis of the viruses revealed that the ratio of NA/HA activities in oseltamivir-resistant viruses was reduced considerably compared to those in wild-type ones.

1. Introduction

Influenza viruses cause annual epidemics in many countries and occasional worldwide pandemics. It was estimated that 250,000–500,000 deaths are associated with influenza epidemics every year (WHO, 2009a). Oseltamivir, one of the marketed influenza virus neuraminidase inhibitors, has been used for the treatment and prophylaxis of influenza and was stockpiled for pandemic influenza. The incidence of oseltamivir-resistant virus varied from 0.3 to 18% during the clinical trials (Ward et al., 2005; Whitley et al., 2001) and the oseltamivir-resistant influenza viruses were detected rarely among circulating viruses before 2007 (WHO, 2007). From the season of 2007–2008, oseltamivir-resistant seasonal influenza A (H1N1) viruses were detected in Europe and spread globally (Hauge et al., 2009; Hurt et al., 2009a; Meijer et al., 2009). By the season of 2008–2009, most isolated seasonal influenza H1N1 strains were found to encode the H275Y substitution in the NA gene, conferring resistance to oseltamivir (WHO, 2010a). Emergence and spread of oseltamivir-resistant viruses pose a concern regarding the strategies for treating and preventing influenza.

An influenza outbreak caused by swine-origin influenza A (H1N1) viruses was detected initially in Mexico and USA in March–April 2009 (CDC, 2009a). The viruses spread rapidly and caused the first influenza pandemic of the 21st century. This pandemic (H1N1) 2009 influenza virus was sensitive to oseltamivir but resistant to the M2 inhibitors amantadine and rimantadine (CDC, 2009b). Since the first oseltamivir-resistant virus was reported in June 2009, an additional 225 cases out of more than 20,000 specimens were reported and confirmed worldwide as of February 2010 (WHO, 2010b). Sixteen cases of these oseltamivir-resistant viruses had no known association with oseltamivir treatment and seven were identified as a cluster with epidemiological linkage (Bai et al., 2009; Mai et al., 2009; WHO, 2010b). This finding raises a concern that oseltamivir-resistant pandemic (H1N1) 2009 influenza virus might spread and become predominant.

* Corresponding author.
** Corresponding author at: National Influenza Center, Centers for Disease Control, No. 161, Kun-Yang Street, Taipei 11561, Taiwan, ROC.
Tel.: +886 2 2653 1108; fax: +886 2 2785 3944.
E-mail addresses: wuhs@cdc.gov.tw (H.-S. Wu), mtliu@cdc.gov.tw (M.-T. Liu).

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In order to investigate the frequency of oseltamivir-resistant pandemic (H1N1) 2009 influenza A viruses in Taiwan, we collected the pandemic H1N1 virus-positive original specimens as well as the respective isolates after culture and analyzed the sequences of their NA genes. In addition, to understand the developmental process of oseltamivir-resistant pandemic H1N1 viruses, we analyzed the clinical specimens taken at different time-points, including those collected before or after oseltamivir treatment as well as their respective cultured isolates. The findings in the present study highlight the need for intensive surveillance of resistant viruses and the importance of direct gene analysis from clinical specimens for drug resistance surveillance.

2. Materials and methods

2.1. Collection of specimens and viral isolates

This study was conducted within the frame of the national influenza surveillance in Taiwan. Influenza isolates or original specimens from hospitalized patients, outpatients in communities, and clustering patients were collected and submitted to Taiwan CDC. In general, throat and nasal swabs were collected from patients who had influenza-like illness with or without severe complications and transported to the laboratories of the influenza surveillance network in Taiwan for analysis. From May 2009 to January 2010, of the 1187 pandemic H1N1 virus-positive cases, 604 were from hospitalized patients, 402 were from outpatients in communities, and 171 were from clusters. Among them, eight cases from hospitalized patients were found to encode the H275Y substitution in the NA gene in their original specimens. Amino acid sequences were predicted from the viral NA nucleotide sequences spanning position 275 following conventional RT-PCR and eight cases were characterized as oseltamivir-resistant by the presence of a tyrosine codon (TAC) at position 275 (N1 numbering). When the case was positive for the H275Y substitution, an epidemiological investigation was undertaken to determine the source of resistant viruses and whether further transmission had occurred. All of the H275Y-positive specimens were collected from patients who had been treated with oseltamivir. To express each of the original specimens and the respective cultured isolates from the eight cases more clearly and easily, we also used representative codes, comprised by case number, the nature of the preparation (original specimens or cultured isolates, denoted as O or C, respectively), the day of collection, and the amino acid sequence at position 275 in the NA gene. In case 1, for example, the oseltamivir-sensitive virus, A/Taiwan/6662/2009, isolated from day 1 could be represented as case 1-C1H and the resistant one, A/Taiwan/6663/2009, isolated from day 4 as case 1-C4Y. Similarly, in case 7, the virus in the original specimen collected at day 6, and the respective cultured isolate, A/Taiwan/7949/2009, harbored the mixture of the 275H and 275Y in NA gene, were denoted as case 7-O6H/Y and case 7-C6H/Y, respectively.

2.2. RNA extraction and identification of pandemic H1N1 viruses

Viral RNA was extracted from 140 μl of the supernatant of original swab specimens and cultured viral isolates using QIAamp Viral RNA Mini Kits, according to the Manufacturer's instructions (Qiagen, Santa Clara, CA). Automated extraction also was conducted using the MagNa Pure LC extraction system (Roche). Extracted RNA was first analyzed to determine the presence of pandemic H1N1 viruses by one-step real-time RT-PCR (Yang et al., 2009). Oseltamivir resistance was determined by conventional RT-PCR using QIAGEN OneStep RT-PCR Kit (Qiagen) and primers published by WHO spanning position 275 (N1 numbering, 274 in N2 numbering) of the neuraminidase (NA) gene (NA-536F 5′-GGTCAGCAAGCGCWTGYCATGA-3′ and NA-1326R 5′-GCTGCTYCCRCTAGTCCAGAT-3′) (WHO, 2009b). Nucleotide sequences of the amplified PCR products were determined and used for further analysis.

2.3. Genetic analysis of the pandemic H1N1 virus

The full-length sequences of HA and NA genes of the virus isolates encoding the H275Y substitution also were determined using the primers and protocol published by WHO (WHO, 2009b). To provide a more detailed comparison of the genetic characteristics and relationship of the oseltamivir-resistant viruses in our study, the full-length sequences of the HA and NA genes were analyzed together with two sets of the selected reference strains. The first set comprised the dominant oseltamivir-sensitive pandemic H1N1 isolates defined by the number of virus isolates with more than 20 (for HA) or 10 (for NA) identical amino acid sequences of HA and NA genes submitted to GenBank before March 25, 2010. Selected based on the HA genes were A/New York/31/2009 (n = 469), A/Mexico/4115/2009 (n = 305), A/Canada-QC/RV1595/2009 (n = 53), A/California/VRDL61/2009 (n = 52), A/Australia/1/2009 (n = 27), A/California/05/2009 (n = 23) and A/Wisconsin/629-S1384/2009 (n = 22). Selected for the NA genes were A/Mexico/4482/2009 (n = 1007), A/California/04/2009 (n = 212), A/California/05/2009 (n = 52), A/Louisiana/03/2009 (n = 24), A/Pennsylvania/10/2009 (n = 13), A/Michigan/02/2009 (n = 11) and A/Toronto/3184/2009 (n = 10). The second set comprised all of the oseltamivir-resistant viruses with the full-length HA and NA genes available in GenBank, including A/Denmark/528/2009 (n = 9), A/Osaka/180/2009, A/Washington/29/2009, A/Washington/28/2009, A/Texas/47/2009 and A/Nagasaki/HA-58/2009. The vaccine strain A/California/07/2009 also was included. Multiple sequence alignments, protein translation and phylogenetic analysis were performed on the basis of nucleotide sequences using the software MEGA4 (Tamura et al., 2007) and BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). A phylogenetic tree was constructed by the neighbor-joining method and 1000 bootstrap replications were performed to evaluate the reliabilities.

2.4. NA inhibition assay of the cultured pandemic H1N1 virus isolates

The 50% inhibitory concentration (IC_{50}) analysis of oseltamivir and zanamivir for the pandemic H1N1 isolates was determined using the NA-Star Influenza Neuraminidase Inhibitor resistance Detection Kit (Applied Biosystems) according to the Manufacturer's recommendations. Briefly, 25 μl of half-log dilutions of neuraminidase inhibitor (NI) from 0.03 to 1000 nM were mixed with 25 μl of virus dilution with a hemagglutination titer greater than or equal to 16 in each well of a white 96-well microplate and incubated 10–20 min at 37 °C. Two wells of the mixture destined to be negative controls contained only assay buffers, instead of NI, and culture medium, instead of virus, also were included. Then, 10 μl of diluted substrate was added to each well and incubated for 10–30 min at room temperature, followed by the addition of 60 μl of accelerator, the chemiluminescent signal being measured immediately. The software GraphPad Prism version 4.00 was used to determine the IC_{50} values. For NA enzyme activity determination, 25 μl of virus dilution was mixed with 25 μl of assay buffers, instead of NI. One well of negative control contained assay buffers and culture medium also was included. All of the experiments of IC_{50} and NA enzyme activity determination were done with three replicates.

Table 1
Basic information of the patients infected by pandemic H1N1 viruses with H275Y substitutions.

Case no.	Age	Gender	Inhabited region	Illness onset (in 2009)	Duration (days) from Tamiflu administration to mutation detection	Duration (days) of hospitalization	Pneumonia during hospitalization
Case 1	22	Male	Kaohsiung	Sep. 1	4	21	Yes
Case 2	45	Male	Taoyuan	Aug. 28	8	5	Yes
Case 3	3	Female	Taoyuan	Oct. 10	6	7	Yes
Case 4	6	Female	Taoyuan	Oct. 24	5	4	Yes
Case 5	13	Female	Taichung	Nov. 3	2	6	Yes
Case 6	1	Female	Pingtung	Oct. 31	12	60	No
Case 7	12	Female	Taipei	Nov. 18	6	5	Yes
Case 8	8	Male	Tainan	Dec. 5	5	13	No

2.5. Sequences information

The HA and NA gene sequences of influenza viruses in this study have been submitted to GenBank and their accession numbers are HM072403–HM072426.

3. Results

3.1. Detection of the H275Y mutation associated with oseltamivir resistance in the NA genes of pandemic (H1N1) 2009 influenza A viruses

From May 2009 to January 2010, a total of 1187 pandemic H1N1 virus-positive samples from hospitalization, community surveillance and outbreak cases in Taiwan were tested for the mutation generating H275Y in the NA gene, conferring resistance to oseltamivir. Basic information from the eight positive cases was reviewed (Table 1), as well as the clinical history, including day of illness onset, specimen collection and treatment with oseltamivir (Table 1 and Fig. 1). These eight cases were reported from hospital-based surveillance and were diagnosed with pneumonia, except for cases 6 and 8. The case 6 was an immunodeficient patient with respiratory and gastrointestinal diseases and case 8 had underlying acute lymphoblastic leukemia and high risk precursor B-cell on post chemotherapy. In the eight cases, viruses encoding the H275Y substitution emerged with a range of 2–12 days from oseltamivir administration to detection of the mutation. They recovered eventually after 4–21 days of hospitalization, except for case 6 who was hospitalized with another disease and exhibited prolonged shedding of the virus for more than 2 months (Table 1 and Fig. 1). The epidemiologic investigation indicated that each of the cases was detected sporadically and no further transmission was found after the screening of suspected cases with geographic proximity and temporal association. The three of the eight patients (cases 1, 6 and 8) had paired clinical specimens taken separately before and after oseltamivir administration and the other five had only specimens collected after drug treatment (Fig. 1). Because all of the specimens encoding the H275Y substitution were detected after oseltamivir treatment (Fig. 1) and those collected before oseltamivir treatment from the three cases were wild-type, it indicated the H275Y substitution arose de novo in cases 1, 6 and 8 after drug treatment.

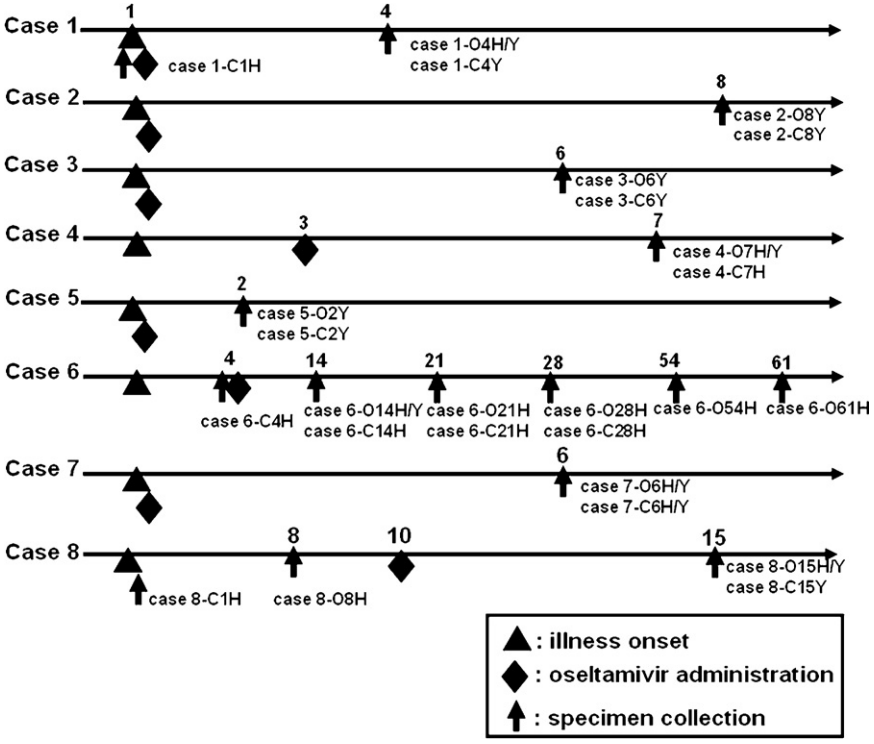

Fig. 1. Timelines of the eight cases infected by pandemic (H1N1) 2009 influenza A viruses encoding H275Y substitution in the NA gene. The time-points of onset of illness, oseltamivir administration and specimen collection for each case are indicated by symbols, as shown in the key. The day of onset of illness was counted as day 1 and durations from onset to respective time-point are shown by the numbers above the line. The viruses, denoted by the representative codes (described in Section 2) are shown below the line.

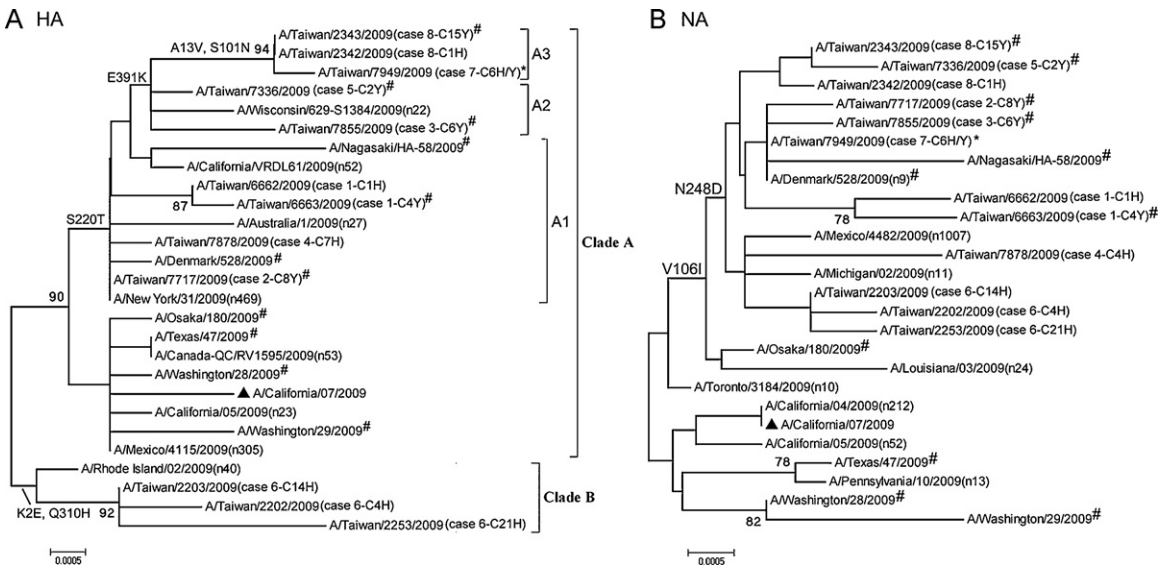

Fig. 2. Phylogenetic relationships of the (A) HA and (B) NA genes of pandemic (H1N1) 2009 influenza A viruses. The phylogenetic analyses were conducted using the neighbor-joining method with 1000 bootstrap replications. Branch values of more than 75 are indicated. The vaccine strain, A/California/07/2009, is indicated by bold triangles. The number of virus isolates with identical full-length amino acid sequences is given following the names of the viruses. For example, A/Mexico/4115/2009 (n305) indicated there were 305 isolates carrying the same amino acid sequences of the HA gene as A/Mexico/4115/2009. All of the viruses from the eight cases were illustrated as virus name accompanying their representative codes. Symbols denote: #, H275Y mutation in NA genes; *, mixed 275H/Y.

The specimens encoding the H275Y substitution in NA genes were then cultured and the resultant isolates also were subjected to sequence analysis, as shown in Fig. 1. Of note, based on the nucleotide sequences of the original specimens, there were five cases (cases 1, 4, 6, 7 and 8) containing mixed populations (T/C) at the first nucleotide of the codon, which determined the amino acid at position 275 as histidine (H), as in the oseltamivir-sensitive dominant virus, or tyrosine (Y), as in oseltamivir-resistant mutant virus (Fig. 1). After the specimens were inoculated onto MDCK cells, the isolates of case 1-C4Y and of case 8-C15Y had a tyrosine at position 275 in the NA gene and the other three had different patterns in that, in cases 4 and 6, all of the cultured isolates encoded histidine and the virus from case 7 remained mixed, with tyrosine and histidine encoded at that position (Fig. 1).

3.2. Genetic analysis of the viruses from the eight cases

Based on the phylogenetic analyses of HA genes, the viruses from the eight cases could be classified into two clades, A and B (Fig. 2). The viruses from cases 1, 2, 3, 4, 5, 7 and 8 clustered in the larger clade A with most of the reference strains and case 6 fell into clade B with the reference strain A/Rhode Island/02/2009. The viruses from the seven cases in clade A could be divided further into three subclades (A1–3) according to the substitutions A13V, S101N, S220T and E391K (Fig. 2). The viruses of case 1-C1H, case 1-C4Y, case 2-C8Y and case 4-C7H were located at the first subclade A1, with the S220T substitution; the case 3-C6Y and case 5-C2Y isolates fell into subclade A2, with the S220T-E391K substitutions as well as the case 7-C6H/Y, case 8-C1H and case 8-C15Y were closely clustered in a distinguishable subclade A3, with the A13V-S101N-S220T-E391K substitutions. Based on the phylogenetic analysis of the NA gene, the viruses of all cases were located at a distinguishable clade with N248D substitution (Fig. 2).

The comparison of the amino acid substitutions between various counterparts is shown in Table 2. From the five paired samples (cases 2, 5, 6, 7 and 8), for HA sequences, three (cases 2, 6 and 8) were found to have identical viral sequences and another two had changes: A26T in case 5 and A232E in case 7 by comparing the cultured isolates with the respective original specimens (Table 2). For the NA genes, there was no difference between the paired sequences. By comparing the viruses isolated after and before oseltamivir treatment from the cases 1, 6 and 8, A232V in case 1-

Table 2
Amino acid substitutions of HA and NA genes between various counterparts.

Sources of the viruses	Amino acid substitutions					
	HA gene			NA gene		
	A[a]	B[b]	C[c]	A	B	C
Case 1	ND[d]	A232V	A232V	ND	S52N, M359I	S52N
Case 2	–[e]	ND	–	–	ND	–
Case 3	ND	ND	V7I, F217L, E391K	ND	ND	–
Case 4	ND	ND	K39R,	ND	ND	I211V
Case 5	A26T	ND	A26T, E391K	–	ND	–
Case 6	–	I119R, T343S	K2E, T220S, Q310H	–	–	–
Case 7	A232E	ND	A13V, S101N, A232E, E391K	–	ND	–
Case 8	–	–	A13V, S101N, E391K	–	–	–

[a] Compared the viruses in original specimens of each case with their respective cultured isolates.
[b] Compared the viruses of each case isolated after with those before oseltamivir administration.
[c] Compared the respective cultured isolates of each case with A/Denmark/528/2009.
[d] Original specimens or full length sequences were not available.
[e] No changes.

C1Y as well as I119R and T343S in case 6-C14H and case 6-C21H were detected in HA sequences (Table 2). For the NA genes, only case 1-C4Y showed S52N and M359I changes. With reference to the oseltamivir-resistant virus, A/Denmark/528/2009, several substitutions were observed in the HA genes, including A232V in case 1-C4Y, V7I, F217L and E391K in case 3-C6Y, K39R in case 4-C7H, A26T and E391K in case 5-C2Y, K2E, T220S and Q310H in case 6-C3H, case 6-C14H and case 6-C21H, A13V, S101N, A232E and E391K in case 7-C6H/Y, as well as A13V, S101N, and E391K in case 8-C1H and case 8-C15Y (Table 2). For the NA genes, substitutions of S52N in case 1-C4Y and I211V in case 4-C7H were found (Table 2).

3.3. Neuraminidase activity of the viruses encoding 275H, 275Y or 275Y/H in NA gene

The 50% inhibitory concentrations (IC_{50}) for oseltamivir and zanamivir against the cultured viruses from the eight cases were determined (Table 3). Of these isolates, the oseltamivir IC_{50} values of case 1-C4Y, case 2-C8Y, case 3-C6Y, case 5-C2Y and case 8-C15Y were in the range 99.1–131.4 nM, while the values of case 4-C7H and case 6-C3H, case 6-C14H and case 6-C21H were 0.4–0.6 nM. However, the oseltamivir IC_{50} value of case 7-C6H/Y was 1.00 nM and similar to the wild-type. It implied that, for pandemic H1N1 virus, the oseltamivir-resistant virus mixed with wild-type viruses will be missed if detection is attempted using enzymatic methods, such as chemiluminescent-based NA inhibition assays. In addition, the zanamivir IC_{50} values against the cultured isolates was in the range 0.7–1.83 nM (Table 3), indicating that all of them were sensitive to zanamivir. We also determined the NA enzymatic activity of the viruses from the eight cases with an equal amount of 16 HA units and found that those of the viruses with H275Y mutation were decreased to the level of 9–33%, compared to wild-type viruses (Fig. 3). These results indicate that the ratio of NA/HA activity in pandemic H1N1 viruses was impaired by the H275Y substitution.

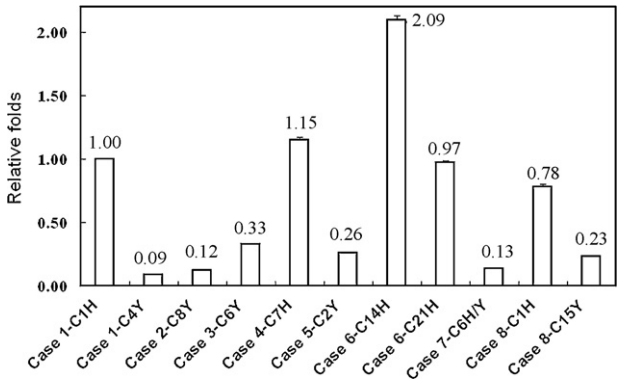

Fig. 3. Neuraminidase activities of the viruses. The neuraminidase activities of the cultured isolates at the equal amount of 16 HA units were determined by chemiluminescent-based NA assays. The enzyme activity of A/Taiwan/6662/2009 was set to 1 and the relative fold increases or decreases of the other viruses were calculated.

4. Discussion

In this study, we show a complete analysis of oseltamivir resistance of pandemic H1N1 influenza viruses in Taiwan. Because antiviral drug-resistant variants might arise during propagation in MDCK cells (Hurt et al., 2009b; Okomo-Adhiambo et al., 2010), we analyzed directly the specimens taken at different time-points as well as their respective cultured isolates. When focused on the amino acid position 275, mixed populations (275H/Y) of NA genes were detected in the original specimens in five of the eight cases (cases 1, 4, 6, 7 and 8). After culturing in MDCK cells, these virus isolates became 275Y-type (cases 1 and 8), 275H-type (cases 4 and 6) and remained mixed (case 7). As the emergence of the dominant 275H virus was considered to represent adaptation, the 275Y viruses of case 4 and 6 seemed to exhibit less fitness in MDCK cells, although here was also the opposite situation, observed in case 1 and 8, where the 275Y viruses seemed to have replicated better than the 275H viruses in MDCK cells. This kind of viruses showed poorer competition with the wild-type, and likely were not detected and persisted in evolving in the human population. As the oseltamivir-resistant virus may not be detected after culture, the importance of direct gene analysis from clinical specimens for drug-resistance surveillance is therefore highlighted.

The HA genes of the viruses from the eight cases in the present study were divided into four subclades by phylogenetic analysis. They envolved from clade A1 to A3 during August–December 2009 in Taiwan (Fig. 2). However, all of the NA genes from these viruses fell into an undistinguishable single clade. The viruses from cases 2, 3, 5, 6, 7 and 8 encoded the same amino acid sequences in their NA genes, which were also found in nine other references sequences of oseltamivir-resistant viruses deposited in GenBank from various regions before March 25, 2010, including A/Denmark/528/2009, A/Hong Kong/2369/2009, A/Hunan/SWL3/2009, A/Iwate/3/2009, A/Quebec/147365/2009, A/Russia/61/2009, A/Tokushima/2/2009, A/Yamaguchi/22/2009 and A/Singapore/GN285/2009. Among the references, there were two viruses with a documented clinical history; A/Hong Kong/2369/2009 was isolated from a patient without oseltamivir-treatment and the original specimen showed mixed populations (H/Y) at amino acid position 275 of the NA gene (Chen et al., 2009) and A/Quebec/147365/2009 emerged during prophylaxis (Baz et al., 2009). The results indicated that viruses carrying such NA sequences have the potential to develop oseltamivir resis-

Table 3
The IC_{50} value of oseltamivir and zanamivir determined from various virus isolates.

Sources of the viruses	Viral isolates	Representative codes	Date collected (in 2009)	NAI IC_{50} (nM)	
				Oseltamivir carboxylate	Zanamivir
Case 1	A/Taiwan/6662/2009	Case 1-C1H	Sep. 1	0.5	1.4
Case 1	A/Taiwan/6663/2009	Case 1-C4Y	Sep. 4	123.6	1.3
Case 2	A/Taiwan/7717/2009	Case 2-C8Y	Sep. 4	131.4	1.0
Case 3	A/Taiwan/7855/2009	Case 3-C6Y	Oct. 15	99.1	1.3
Case 4	A/Taiwan/7878/2009	Case 4-C7H	Oct. 30	0.6	1.5
Case 5	A/Taiwan/7336/2009	Case 5-C2Y	Nov. 5	123.1	1.2
Case 6	A/Taiwan/2202/2009	Case 6-C3H	Nov. 3	ND[a]	ND
Case 6	A/Taiwan/2203/2009	Case 6-C14H	Nov. 13	0.4	0.9
Case 6	A/Taiwan/2253/2009	Case 6-C21H	Nov. 20	0.6	1.0
Case 7	A/Taiwan/7949/2009	Case 7-C6H/Y	Nov. 23	1.0	0.7
Case 8	A/Taiwan/2342/2009	Case 8-C1H	Dec. 5	0.5	1.2
Case 8	A/Taiwan/2343/2009	Case 8-C15Y	Dec. 19	116.9	1.83

[a] Not determined.

tance and exhibit great fitness in MDCK cells, and that various resistant viruses with combinations of diverse HA genes and a dominant NA gene continue to evolve under the selection pressure of the drug. Of note, the viruses from case 6, who was an immunodeficient patient and remained positive for pandemic H1N1 influenza virus after a course of oseltamivir treatment, exhibited mixed 275H/Y in the clinical specimen and the 275H-viruses emerged alone after virus culture as well as in the following clinical specimens collected after drug treatment was stopped. The HA sequences of them all encoded the substitutions K2E, T220S and Q310H, signatures that classified the viruses into clade 6 (Nelson et al., 2009). This kind of H275Y virus like case 6-O14H/Y which harbored the HA gene belonging to clade 6 in combination with the dominant NA gene seemed unable to adapt in vivo and in MDCK cells. In addition, another poorly adaptive virus also was observed in case 4-O7H/Y virus which had substitutions K39R in the HA gene and I211V in the NA gene (Table 2). The makeup of the HA and NA genes played an important role in determining the viral adaptation in vivo and MDCK cells and some strains with particular genetic backgrounds may be prone to become oseltamivir-resistant.

Previous studies have reported that the H275Y substitution in the N1 subtype is the most common change conferring oseltamivir resistance and has been reported not only in seasonal H1N1 viruses but also in H5N1 viruses (de Jong et al., 2005; Gubareva et al., 2002; Lackenby et al., 2008b; Le et al., 2005). However, other amino acid substitutions in seasonal H1N1 and H5N1viruses, especially those located in and around the NA active site, such as V116, I117, E119, Q136, K150, D151, Q199 and I223, have also been shown to reduce the susceptibility of viruses to NAIs, including oseltamivir and zanamivir (Hurt et al., 2007, 2009b; Lackenby et al., 2008a; Sheu et al., 2008). In our study, changes of these residues were not found in the viruses with the H275Y substitution either in original specimens or the respective cultured isolates. By comparing the amino acid sequences of HA and NA genes from original specimens and the respective cultured isolates, no common residue change was observed (Table 2). Another comparison of the sequences from viruses collected after and before oseltamivir treatment showed that substitutions of A232V in case 1-C4Y and I119R and T343S in case 6-C14H and -C20H viruses for the HA genes and S52N and M359I in case 1-C4Y for the NA genes were observed. We also compared our sequences of HA and NA genes with those submitted to NCBI and found that co-substitutions A13V and S101N in HA genes of the virus from cases 7 to 8 were newly emerged and were not present in GenBank before March 25, 2010. The effects of these substitutions remain unknown. They did not affect the antigenicity, because the isolates from the eight cases were antigenically related to vaccine strain A/California/07/2009 virus (data not shown).

In this study, the IC_{50} values of pandemic H1N1 isolates for oseltamivir correlated with the H275Y substitution in the NA gene, except for the unexpected result obtained from case 7-C6H/Y virus, which showed the IC_{50} for oseltamivir similar to that of the wild-type (Table 3). Further investigation revealed that the NA activity in oseltamivir-resistant viruses was reduced to 9–33% of wild-type viruses (Fig. 3). This raises an important issue that the H275Y substitution upset the balance between HA and NA activities in pandemic H1N1 viruses. The situation may resemble the case of seasonal influenza A (H1N1) viruses, at the early stage before 2007, the H275Y substitution impaired viral fitness and then a set of mutations resulted in a genetic background that overcame the effect of H275Y, such as seen in A/Brisbane/59/2007-like viruses. If the developmental process of oseltamivir-resistant viruses is similar, at the present time, the pandemic H1N1 virus has not yet overcome the obstacle caused by the H275Y substitution. This observation was also mentioned by Bloom et al. (2010) that H275Y substitution decreases the amount of neuraminidase protein that reaches the cell surface in A/California/7/2009 (Bloom et al., 2010). However, in the short period from August to December 2009, we have observed the emergence of heterogeneous oseltamivir-resistant viruses, accompanying the evolution of HA genes from clade A1 to A3. HA genes, as well as NA genes continue evolving and a specific combination of HA and NA substitutions may arise to overcome the impact of the H275Y substitution. Because a concern may be raised that pandemic H1N1 will behave like the seasonal H1N1 viruses in 2008–2009, and become oseltamivir-resistant and spread globally, it is important for influenza antiviral drug-resistant surveillance to monitor the mutation of viral genes from original specimens and the respective cultured isolates, as well as the changes of the NA/HA enzyme activities.

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