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Original Article

Genetic Analysis and Phylogenetic Characterization of Pandemic (H1N1) 2009 Influenza Viruses that Found in Nagasaki, Japan

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SUMMARY: Isolation and determination of the nucleotide sequence of hemagglutinin (HA) of the pandemic (H1N1) 2009 influenza viruses found in Nagasaki, Japan, were conducted. The alignment results of the predicted HA amino acid sequences of these strains compared to the known global isolates revealed 5 specific amino acid differences located within the antigenic sites. The phylogenetic analyses revealed that the majority of the Nagasaki isolates could be classified into 6 phylogenetic clusters. Almost all isolates collected in the early season were classified into cluster I, which apparently originated from A/Nagasaki/HA-6/2009 isolated from a patient who returned from the Philippines. This cluster ceased to spread after November 2009. Between the end of August 2009 and January 2010, 5 new phylogenetic clusters (II–VI) emerged with viruses from different origins, and cluster III continuously advanced until March 2010. These results suggest that the onset of the influenza epidemic in Nagasaki originated from patient(s) who returned from the Philippines, and subsequently, various imported strains from different origins sustained the virus spread. Among the Nagasaki isolates, A/Nagasaki/HA-58/2009 having an H275Y mutation in the neuraminidase gene, which confers resistance to oseltamivir, was isolated. This is the first report in which an oseltamivir-resistant pandemic H275Y mutant was identified in Nagasaki Prefecture.

INTRODUCTION

The pandemic (H1N1) 2009 influenza viruses were first identified in Mexico and the United States in mid-April 2009. The virus quickly spread throughout the human population, and the number of patients increased to hundreds of thousands, and hundreds of deaths had been reported by May 5, 2009 (1). Consequently, the World Health Organization (WHO) raised the influenza pandemic alert level to phase 6 on June 11, 2009. In Japan, the Ministry of Health, Labour and Welfare enforced a quarantine strategy on April 28, 2009 for patients with pandemic (H1N1) 2009 influenza (2). In Japan, the first patients with pandemic influenza were identified at the Narita airport among travelers who returned from Canada on May 9, 2009, and many more pandemic influenza patients were subsequently identified (3). In Nagasaki, the Nagasaki Prefectural Government quarantined patients who visited the hospital with symptoms suspicious for influenza starting on May 18, 2009. The first case of pandemic influenza in Nagasaki was identified among travelers who returned from foreign countries on June 16, 2009. However, the spread of the pandemic (H1N1) 2009 influenza viruses in Nagasaki was not detected until July 20, 2009. This implies that the initial quarantine was effective for preventing the spread of the virus. However, on July 20, 2009, the spread of the pandemic (H1N1) 2009 influenza viruses began among the younger individuals who attended the summer festival held in Nagasaki, which was attended by approximately 2,000 people.

The influenza A virus surface glycoprotein hemagglutinin (HA), which plays a major role in viral attachment and evasion from neutralizing antibody responses, is under selective pressure to mutate to escape from the host immune system. The HA protein consists of 2 domains: the globular head (composed of most of the antigenic HA1 polypeptide of HA) and the long fibrous stem (comprised mostly of the HA2 polypeptide) (4,5). The HA1 domain of the influenza HA protein is the most rapidly evolving region of these viruses. The HA1 domain in H1-subtype strains has 4 additional antigenic sites, including Sa, Sb, Ca, and Cb, that are determined on the basis of the assessment of antigenic variations of virus mutants (4–6). Point mutations in this domain may induce antigenic changes (7,8), known as antigenic drift. Because of the high morbidity and mortality due to influenza epidemics, monitoring of the accumulated antigenic variations in circulating influenza viruses is important for predicting epidemics, severity, and for the design of future vaccines.

The other influenza A virus surface glycoprotein, neuraminidase (NA) (9), which is composed of a tetramer comprising a bulky head attached to a stalk, plays a major role in allowing the release of progeny viruses from an infected cell's surface by digestion of the terminal sialic acids from glycoconjugates on both cell receptors and the viral HA proteins. Thus far, sever-

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al NA inhibitors, including zanamivir and oseltamivir, which bind to the active site of NA, have been produced and used for the treatment of influenza-infected patients (10). However, oseltamivir-resistant influenza virus mutants have been identified in A-type as well as B-type viruses isolated from patients (11–13). Among these viruses, mutants of NA possessing H275Y (substitution of histidine by tyrosine at residue 275 in the NA protein) were identified, which showed high tolerance to oseltamivir (12,13). The spread of oseltamivir-resistant seasonal H1N1 influenza viruses was very rapid, resulting in oseltamivir resistance in more than 90% of the seasonal H1N1 influenza viruses isolated during the 2008/2009 season (14).

In Japan, seasonal H1N1 influenza viruses carrying the above-mentioned mutation were identified among 2.6% of the isolated influenza viruses during the 2007/2008 season; however, more than 99% of the viruses identified during the 2008/2009 season were oseltamivir resistant (14). During the pandemic (H1N1) 2009, oseltamivir-resistant pandemic strains were also rarely identified in some foreign countries and in Japan (15). It can be easily speculated that the oseltamivir-resistant pandemic (H1N1) 2009 influenza viruses may spread rapidly at any time. Therefore, monitoring the possible emergence of oseltamivir-resistant viruses is important.

To characterize the properties of the pandemic influenza viruses in Nagasaki, we analyzed the HA sequences of Nagasaki isolates. Furthermore, we aimed to determine how the pandemic influenza viruses spread in Nagasaki. This analysis can provide a good simulation for estimating the spread of newly emerging pandemic influenza viruses in a region far from the pandemic origin. In the present study, we conducted sequencing and phylogenetic analyses of the HA genes of the pandemic (H1N1) 2009 influenza viruses isolated from patients in Nagasaki (collected from July 2009 to March 2010). We also analyzed partial nucleotide NA sequences, including the H275Y coding region, to identify oseltamivir-resistant mutants. Our data clearly show that the initial quarantine strategy was effective but insufficient to completely prevent the spread of the pandemic (H1N1) 2009 influenza viruses in Nagasaki. Moreover, only one oseltamivir-resistant H275Y mutant was identified during the pandemic season in Nagasaki, and no additional spreading of the virus occurred during the time of our analysis.

MATERIALS AND METHODS

Clinical samples and virus isolation: Nasopharyngeal or throat swab specimens from influenza patients were collected between July 2009 and March 2010 at the Nagasaki Municipal Public Health and Environment Laboratory. The patients were confirmed to be infected with the pandemic (H1N1) 2009 influenza virus by immunochromatography and polymerase chain reaction (PCR) (Table 1). The total number of specimens obtained was 94, and of these, 88 were confirmed to be positive for the pandemic (H1N1) 2009 influenza virus. Isolation of the virus was achieved from 75 positive specimens. The details of these 75 patients are summarized in Table 1. The age of the patients ranged from 0 to 63 years. Of these patients, 36 were men and 39 were women. In patient HA-58, aggravated symptoms despite treatment with oseltamivir were recorded. Patients who had symptoms after participating in the summer festival held in Nagasaki on July 20, 2009, are indicated by boxes in Table 1.

Specimens were filtered, and the resulting samples were inoculated onto Madin-Darby Canine Kidney (MDCK) cells with trypsin. Upon observation for characteristic cytopathic effects in MDCK cells, the virus-containing supernatants were collected. The virus isolation rates in the early (collected between July and August, isolates stored at 4°C) and late (collected between January and March, isolates stored at −80°C) pandemic seasons were high (85.3 and 87.1%, respectively), whereas the rate in the mid-pandemic season (collected between September and December, isolates stored at 4°C) was slightly lower (58.8%) (data not shown). The isolated viruses were named on the basis of their collection numbers as summarized in Table 1.

Genomic sequence analysis: Viral RNAs were extracted from the culture supernatants or swabs using a QIAamp Viral RNA Mini Kit (Qiagen, Valencia, Calif., USA). The resulting RNA was subjected to cDNA synthesis using HA primer 1 (5′-TATTCGTCTCAGGGA GCAAAAGCAGGDKB-3′) and M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, Calif., USA). Subsequently, the resulting cDNA was used for PCR, which was performed in a buffer containing the respective primer sets with *Pfu* DNA polymerase (Bioacademia, Osaka, Japan) to amplify the HA genes. Two primer sets for the 5′-terminal one-third of HA (HA primer 1 and HA primer 2, 5′-CAGCACTAGTAGATGGATG G-3′) and for the 3′-terminal two-thirds of HA (HA primer 3, 5′-ACCCAAAGCTCAGCAAATCC-3′, and HA primer 4, 5′-ATATCGTCTCGTATTAGTAGAA ACAAGGKKSTT-3′) were used. Amplicons of 515 bp and 1,199 bp, respectively, were amplified. For the amplification of partial NA genes, including a region corresponding to the H275Y mutation, PCR was performed using cDNA obtained as described above and a primer set for NA (NA primer 1, 5′-ATCGAACCCTAAT GAGC-3′ and NA primer 2, 5′-TGCATATGTAT CCTATCTG-3′), resulting in a 492-bp amplicon. Direct sequencing of these amplicons was performed using an Applied Bio 3100-Avant Genetic Analyzer (Applied Biosystems, Foster City, Calif., USA).

Phylogenetic tree analysis: We analyzed the phylogenetic relationship of the nucleotide sequences of HA (complete open reading frame) of 75 viruses collected in Nagasaki and compared it with those from global isolates collected between July 2009 and March 2010 by applying the maximum-likelihood method using the phyML software program (16,17). The model, branch support, tree searching operations, and starting tree parameters for the phyML software program were set to GTR, bootstrap with 1,000 replicates, NNI, and BioNJ, respectively. Bootstrap values that had a probability value higher than 50% were added in the tree. The HA sequence data from 107 global isolates of the pandemic (H1N1) 2009 influenza viruses collected between July 1, 2009 and March 31, 2010 were obtained from the Influenza Virus Resource Database (NCBI) on May 3, 2010. The genetic divergence in the viruses was very

Table 1. Data of the specimens from influenza patients in Nagasaki City

Specimen (strain)	Date of collection	Age	Sex	Description	Accession no. of HA sequences
A/Nagasaki/HA-4/2009	5/7/2009	29	male	Travel to Indonesia	AB530161
A/Nagasaki/HA-6/2009	13/7/2009	55	male	Travel to Philippines	AB530249
A/Nagasaki/HA-9/2009	23/7/2009	27	male	Travel to Philippines	AB530461
A/Nagasaki/HA-10/2009	23/7/2009	23	male	Participated in Summer festival	AB530462
A/Nagasaki/HA-11/2009	23/7/2009	59	female	—	AB530463
A/Nagasaki/HA-12/2009	24/7/2009	21	female	Participated in Summer festival	AB530464
A/Nagasaki/HA-13/2009	24/7/2009	22	female	Participated in Summer festival	AB530465
A/Nagasaki/HA-14/2009	24/7/2009	29	male	Participated in Summer festival	AB530466
A/Nagasaki/HA-15/2009	24/7/2009	17	female	Participated in Summer festival	AB530467
A/Nagasaki/HA-16/2009	25/7/2009	23	female	—	AB530468
A/Nagasaki/HA-17/2009	27/7/2009	17	female	—	AB530469
A/Nagasaki/HA-18/2009	27/7/2009	18	female	—	AB530470
A/Nagasaki/HA-22/2009	29/7/2009	23	male	—	AB530471
A/Nagasaki/HA-24/2009	29/7/2009	22	male	—	AB535738
A/Nagasaki/HA-25/2009	30/7/2009	7	male	—	AB530472
A/Nagasaki/HA-26/2009	30/7/2009	9	female	—	AB530473
A/Nagasaki/HA-27/2009	30/7/2009	18	female	—	AB530474
A/Nagasaki/HA-28/2009	30/7/2009	6	male	—	AB530475
A/Nagasaki/HA-29/2009	30/7/2009	17	male	—	AB530476
A/Nagasaki/HA-30/2009	30/7/2009	22	female	—	AB535739
A/Nagasaki/HA-31/2009	3/8/2009	15	female	—	AB530477
A/Nagasaki/HA-32/2009	3/8/2009	17	male	—	AB530478
A/Nagasaki/HA-33/2009	3/8/2009	8	male	—	AB530479
A/Nagasaki/HA-34/2009	3/8/2009	3	male	—	AB530480
A/Nagasaki/HA-35/2009	3/8/2009	10	female	—	AB530481
A/Nagasaki/HA-36/2009	5/8/2009	12	female	—	AB530482
A/Nagasaki/HA-37/2009	5/8/2009	15	female	—	AB535740
A/Nagasaki/HA-38/2009	10/8/2009	5	male	—	AB530483
A/Nagasaki/HA-39/2009	10/8/2009	63	female	—	AB530484
A/Nagasaki/HA-40/2009	17/8/2009	6	male	—	AB530485
A/Nagasaki/HA-41/2009	17/8/2009	5	male	—	AB530486
A/Nagasaki/HA-42/2009	20/8/2009	34	female	—	AB530487
A/Nagasaki/HA-44/2009	20/8/2009	6	male	—	AB530488
A/Nagasaki/HA-46/2009	31/8/2009	13	male	—	AB540654
A/Nagasaki/HA-48/2009	10/9/2009	1	female	—	AB535742
A/Nagasaki/HA-49/2009	14/9/2009	3	male	—	AB539047
A/Nagasaki/HA-50/2009	6/10/2009	12	male	—	AB535743
A/Nagasaki/HA-52/2009	9/10/2009	6	female	—	AB535744
A/Nagasaki/HA-53/2009	9/10/2009	10	male	—	AB535745
A/Nagasaki/HA-56/2009	22/10/2009	2 months	female	—	AB539046
A/Nagasaki/HA-57/2009	26/10/2009	13	male	—	AB536768
A/Nagasaki/HA-58/2009	27/10/2009	6	female	Symptoms worsened during treatment with oseltamivir (H275Y mutant)	AB536769
A/Nagasaki/HA-59/2009	27/10/2009	2 months	female	—	AB535746
A/Nagasaki/HA-60/2009	27/10/2009	8	female	—	AB535747
A/Nagasaki/HA-62/2009	29/10/2009	3 months	female	—	AB536770
A/Nagasaki/HA-63/2009	29/10/2009	18	male	—	AB535748
A/Nagasaki/HA-64/2009	27/11/2009	9	female	—	AB558535
A/Nagasaki/HA-65/2009	3/12/2009	56	male	—	AB558536
A/Nagasaki/HA-10-1/2010	4/1/2010	9	male	—	AB551871
A/Nagasaki/HA-10-2/2010	4/1/2010	6	male	—	AB551872
A/Nagasaki/HA-10-3/2010	4/1/2010	9	male	—	AB551873
A/Nagasaki/HA-10-4/2010	4/1/2010	4	female	—	AB551874
A/Nagasaki/HA-10-5/2010	12/1/2010	13	female	—	AB551875
A/Nagasaki/HA-10-6/2010	12/1/2010	2	male	—	AB551876
A/Nagasaki/HA-10-7/2010	12/1/2010	21	female	—	AB551877
A/Nagasaki/HA-10-11/2010	12/1/2010	12	male	—	AB551880
A/Nagasaki/HA-10-9/2010	18/1/2010	0	female	—	AB551878
A/Nagasaki/HA-10-10/2010	18/1/2010	13	female	—	AB551879
A/Nagasaki/HA-10-12/2010	18/1/2010	2 months	female	—	AB551881
A/Nagasaki/HA-10-13/2010	18/1/2010	9	female	—	AB551882
A/Nagasaki/HA-69/2010	20/1/2010	18	male	—	AB551883
A/Nagasaki/HA-10-14/2010	1/2/2010	11	male	—	AB558537
A/Nagasaki/HA-10-15/2010	1/2/2010	13	female	—	AB558538
A/Nagasaki/HA-10-17/2010	8/2/2010	4	female	—	AB558539
A/Nagasaki/HA-10-18/2010	15/2/2010	9	male	—	AB558540
A/Nagasaki/HA-10-19/2010	15/2/2010	6	male	—	AB558541
A/Nagasaki/HA-10-20/2010	15/2/2010	16	female	—	AB558542
A/Nagasaki/HA-10-21/2010	22/2/2010	6	female	—	AB558543
A/Nagasaki/HA-10-22/2010	1/3/2010	12	male	—	AB558544
A/Nagasaki/HA-10-24/2010	8/3/2010	6	female	—	AB558545
A/Nagasaki/HA-10-25/2010	8/3/2010	10	female	—	AB558546
A/Nagasaki/HA-10-26/2010	15/3/2010	4	male	—	AB558547
A/Nagasaki/HA-10-27/2010	15/3/2010	3	female	—	AB558548
A/Nagasaki/HA-10-28/2010	23/3/2010	13	female	—	AB558549
A/Nagasaki/HA-10-30/2010	29/3/2010	15	male	—	AB558550

small, as illustrated by the fact that the bootstrap values for the nodes were in general very low. This applied to most of the phylogenetic analyses of these newly emerged viruses. Hence, we could not use the bootstrap values as references to designate the Nagasaki clusters. Therefore, the designation of the Nagasaki clusters (I to VI) was conducted by connecting the sub-clusters of evolutionarily close Nagasaki isolates.

We also analyzed the phylogenetic relationship of the nucleotide HA sequences of 14 Nagasaki isolates (classified as cluster I) by the above-mentioned maximum-likelihood method and compared it with those of the isolates A/Philippines/2001/2009, Mexican (7 strains), and American (11 strains), which were isolated between April and May 2009 (initially spreading viruses) obtained from the Influenza Virus Resource Database on May 3, 2010.

In vitro oseltamivir-resistance assay: To determine the relative sensitivities of the viruses to oseltamivir in liquid culture, MDCK cells seeded in 96-well plates were challenged with approximately 100 of 50% tissue culture infective dose ($TCID_{50}$) of each virus. Serial 3-fold dilutions of oseltamivir were added to each well in duplicate. The culture plates were incubated for 3 days at 37°C, and the growth of the virus was detected by crystal violet staining and absorbance at a wavelength of 560 nm with an infinite M200 spectrometer (TECAN, Kanagawa, Japan). The concentrations of oseltamivir ranged from 0.2 μM to 1,000 μM.

Nucleotide sequence accession numbers: The nucleotide sequences of HA determined in this study can be found in the DDBJ, EMBL, and GenBank databases under the indicated accession numbers summarized in Table 1.

RESULTS

Sequence analysis and amino acid differences: The nucleotide sequences of all HA genes of the viruses collected from patients in Nagasaki revealed a sequence identity that was higher than 99.5% compared to other pandemic (H1N1) 2009 influenza viruses. These data indicated that the isolated viruses were indeed pandemic influenza viruses. The mean nucleotide divergences (mean ± SE) within Nagasaki isolates and in comparison to the pandemic standard A/California/07/2009 strain were 0.00370 ± 0.00064 and 0.00518 ± 0.00137, respectively. The divergences within each isolation period (July to September, October to December, and January to March) were 0.00136 ± 0.00040, 0.00386 ± 0.00083, and 0.00534 ± 0.00095, respectively (data not shown). These results were analyzed using the maximum composite likelihood method and the MEGA version 4.0 software package with 500 bootstrap replicates (18). The predicted amino acid sequences of HA of the isolated viruses in Nagasaki did not contain a conserved arginine or a stretch of basic amino acids in the cleavage site, whereas a trypsin-like protease cleavage site, which can be cleaved by proteases existing in the respiratory tract (PSIQSR/GLF amino acid positions of 339 to 347), has been identified without any mutations (data not shown). Furthermore, a single potential *N*-glycosylation site (N104) in the globular head region of HA corresponding to one of the antigenic characteristics was completely conserved among these strains, as reported for the pandemic 1918 case, as well as in recent seasonal human (H1N1) viruses. Other potential 4-5 *N*-glycosylation sites (N-Xaa-S/T, where Xaa is any amino acid except P) that have been acquired in several recent seasonal (H1N1) viruses (19) were not found in the pandemic (H1N1) 2009 influenza viruses (data not shown). Amino acid alignment of the HA of Nagasaki isolates against global isolates was performed, and the results are summarized in Table 2. The amino acid differences of the HA of Nagasaki isolates were I4K, D52G, K53N, T106M, S138I, N146D, V149E, A156D, A203T, D204G, A214T, T220S, D239E, A273T, N311D, P314S, P321S, G360E, E391K, N461K, T491P, I527V, and I564K. Among these amino acid differences, D52G, T106M, D239E, P314S, and E391K were also observed in several other foreign isolates (data not shown). A156D, A203T, D204G, T220S, and D239E were located within the antigenic HA1 sites (4–6). In the early pandemic season (July to August 2009), the amino acid differences S138I, T220S, and P321S were mainly observed, and S138I and P321S continued to be observed until October 2009.

Several other amino acid differences (D239E, P314S, and I527V) began to be observed from the end of August. The amino acid differences I4K and A214T began to be observed from the beginning and the end of October 2009, respectively. Amino acid differences in the antigenic sites (4–6) of Ca_2 (A156D in A/Nagasaki/HA-10-4, 5, and 10/2010) and of Sb (A203T in A/Nagasaki/HA-10-13/2010 and D204G in A/Nagasaki/HA-10-5, 6, and 9/2010) were frequently observed in strains collected between January 4 and 18, 2010. From mid-January until the end of February 2010, other amino acid differences (D52G, N146D, V149E, A203T, N311D, E391K, T491P, and I564K) were primarily observed. The variation rate tended to change at an approximately 2-month interval. These strains likely originated outside of Nagasaki.

The analyses of partial NA sequences revealed that an amino acid substitution, H275Y, which is known to confer resistance to oseltamivir, was found in strain A/Nagasaki/HA-58/2009 (Table 1). This strain was the only one that had the mutation among 75 Nagasaki isolates.

Phylogenetic tree analysis: Analysis of the phylogenetic relationship was based on nucleotide sequences of HA of the isolated viruses and carried out by the maximum-likelihood method against sequences from global isolates. Almost all viruses isolated in Nagasaki City could be classified into 6 clusters (I–VI) (Fig. 1). Two strains, A/Nagasaki/HA-4/2009 and A/Nagasaki/HA-9/2009, isolated from patients who returned from abroad, appeared at different positions compared with other Nagasaki isolates. These patients had apparently been infected in foreign countries. Almost all Nagasaki isolates collected during the earlier season (July to October 2009) were classified into cluster I, which evolutionarily originated from A/Nagasaki/HA-6/2009, which was isolated from a patient who returned from the Philippines. The spread of this cluster started after July 20, 2009, and advanced until the end of October of 2009. Sixteen strains in cluster I (A/Nagasaki/HA-11, 13, 24, 27, 30–40, and 42/2009) had

Table 2. The amino acid sequence of HA in the Nagasaki isolates

Strain name	Date of collection	Amino acid diffrences in Nagasaki isolates[1)]																						
		4	52	53	106	138	146	149	156 (a)	203 (b)	204 (b)	214	220 (c)	239 (a)	273	311	314	321	360	391	461	491	527	564
A/New York/3191/2009 (control)	25/4/2009	I	D	K	T	S	N	V	A	A	D	A	T	D	A	N	P	P	G	E	N	T	I	I
A/Nagasaki/HA-4/2009	5/7/2009	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
A/Nagasaki/HA-6/2009	13/7/2009	—	—	—	—	—	—	—	—	—	—	—	S	—	—	—	—	—	—	—	—	—	—	—
A/Nagasaki/HA-9/2009	23/7/2009	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
A/Nagasaki/HA-10/2009	23/7/2009	—	—	N	—	—	—	—	—	—	—	—	—	—	—	—	—	S	—	—	—	—	—	K
A/Nagasaki/HA-11/2009	23/7/2009	—	—	—	—	I	—	—	—	—	—	—	—	—	—	—	—	S	—	—	—	—	—	—
A/Nagasaki/HA-12/2009	24/7/2009	—	—	—	—	I	—	—	—	—	—	—	S	—	—	—	—	S	—	—	—	—	—	—
A/Nagasaki/HA-13/2009	24/7/2009	—	—	—	—	I	—	—	—	—	—	—	—	—	—	—	—	S	—	—	—	—	—	—
A/Nagasaki/HA-14/2009	24/7/2009	—	—	—	—	I	—	—	—	—	—	—	S	—	—	—	—	S	—	—	—	—	—	K
A/Nagasaki/HA-15/2009	24/7/2009	—	—	—	—	I	—	—	—	—	—	—	—	—	—	—	—	S	—	—	—	—	—	—
A/Nagasaki/HA-16/2009	25/7/2009	—	—	—	—	I	—	—	—	—	—	—	S	—	—	—	—	S	—	—	—	—	—	—
A/Nagasaki/HA-17/2009	27/7/2009	—	—	N	—	—	—	—	—	—	—	—	S	—	—	—	—	S	—	—	—	—	—	—
A/Nagasaki/HA-18/2009	27/7/2009	—	—	—	—	I	—	—	—	—	—	—	—	—	—	—	—	S	—	—	—	—	—	—
A/Nagasaki/HA-22/2009	29/7/2009	—	—	—	—	I	—	—	—	—	—	—	S	—	—	—	—	S	—	—	—	—	—	—
A/Nagasaki/HA-24/2009	29/7/2009	—	—	—	—	I	—	—	—	—	—	—	—	—	—	—	—	S	—	—	—	—	—	—
A/Nagasaki/HA-25/2009	30/7/2009	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	S	—	—	—	—	—	—
A/Nagasaki/HA-26/2009	30/7/2009	—	—	—	—	I	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
A/Nagasaki/HA-27/2009	30/7/2009	—	—	—	—	I	—	—	—	—	—	—	—	—	—	—	—	S	—	—	—	—	—	—
A/Nagasaki/HA-28/2009	30/7/2009	—	—	—	—	I	—	—	—	—	—	—	S	—	—	—	—	S	—	—	—	—	—	—
A/Nagasaki/HA-29/2009	30/7/2009	—	—	—	—	I	—	—	—	—	—	—	S	—	—	—	—	—	—	—	—	—	—	—
A/Nagasaki/HA-30/2009	30/7/2009	—	—	—	—	I	—	—	—	—	—	—	—	—	—	—	—	S	—	—	—	—	—	—
A/Nagasaki/HA-31/2009	3/8/2009	—	—	—	—	I	—	—	—	—	—	—	—	—	—	—	—	S	—	—	—	—	—	—
A/Nagasaki/HA-32/2009	3/8/2009	—	—	—	—	I	—	—	—	—	—	—	—	—	—	—	—	S	—	—	—	—	—	—
A/Nagasaki/HA-33/2009	3/8/2009	—	—	—	—	I	—	—	—	—	—	—	—	—	—	—	—	S	—	—	—	—	—	—
A/Nagasaki/HA-34/2009	3/8/2009	—	—	—	—	I	—	—	—	—	—	—	—	—	—	—	—	S	—	—	—	—	—	—
A/Nagasaki/HA-35/2009	3/8/2009	—	—	—	—	I	—	—	—	—	—	—	—	—	—	—	—	S	—	—	—	—	—	—
A/Nagasaki/HA-36/2009	5/8/2009	—	—	—	—	I	—	—	—	—	—	—	—	—	—	—	—	S	—	—	—	—	—	—
A/Nagasaki/HA-37/2009	5/8/2009	—	—	—	—	I	—	—	—	—	—	—	—	—	—	—	—	S	—	—	—	—	—	—
A/Nagasaki/HA-38/2009	10/8/2009	—	—	—	—	I	—	—	—	—	—	—	—	—	—	—	—	S	—	—	—	—	—	—
A/Nagasaki/HA-39/2009	10/8/2009	—	—	—	—	I	—	—	—	—	—	—	—	—	—	—	—	S	—	—	—	—	—	—
A/Nagasaki/HA-40/2009	17/8/2009	—	—	—	—	I	—	—	—	—	—	—	—	—	—	—	—	S	—	—	—	—	—	—
A/Nagasaki/HA-41/2009	17/8/2009	—	—	—	—	I	—	—	—	—	—	—	—	—	—	—	—	S	—	—	—	—	V	—
A/Nagasaki/HA-42/2009	20/8/2009	—	—	—	—	I	—	—	—	—	—	—	—	—	—	—	—	S	—	—	—	—	—	—
A/Nagasaki/HA-44/2009	20/8/2009	—	—	—	—	—	—	—	—	—	—	—	—	E	—	—	S	—	—	—	K	—	—	—
A/Nagasaki/HA-46/2009	31/8/2009	—	—	N	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
A/Nagasaki/HA-48/2009	10/9/2009	—	—	—	—	I	—	—	—	—	—	—	—	—	—	—	—	S	—	—	—	—	—	—
A/Nagasaki/HA-49/2009	14/9/2009	—	—	—	—	I	—	—	—	—	—	—	—	—	—	—	—	S	—	—	—	—	—	—
A/Nagasaki/HA-50/2009	6/10/2009	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
A/Nagasaki/HA-52/2009	9/10/2009	—	—	—	—	—	—	—	—	—	—	—	—	E	—	—	S	—	—	—	—	—	V	—
A/Nagasaki/HA-53/2009	9/10/2009	K	—	—	—	—	—	—	—	—	—	—	—	—	—	—	S	—	—	—	—	—	V	—
A/Nagasaki/HA-56/2009	22/10/2009	—	—	—	—	—	—	—	—	—	—	T	—	—	—	—	—	—	—	—	—	—	—	—
A/Nagasaki/HA-57/2009	26/10/2009	—	—	—	—	—	—	—	—	—	—	T	—	—	—	—	—	—	—	—	—	—	—	—
A/Nagasaki/HA-58/2009	27/10/2009	—	—	—	—	I	—	—	—	—	—	—	—	—	—	—	—	S	—	—	—	—	—	—
A/Nagasaki/HA-59/2009	27/10/2009	—	—	—	—	—	—	—	—	—	—	T	S	—	—	—	—	—	—	—	—	—	—	—
A/Nagasaki/HA-60/2009	27/10/2009	—	—	—	—	I	—	—	—	—	—	—	S	—	—	—	—	S	—	—	—	—	—	—
A/Nagasaki/HA-62/2009	29/10/2009	—	—	—	M	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
A/Nagasaki/HA-63/2009	29/10/2009	—	—	—	—	—	—	—	—	—	—	T	S	—	—	—	—	—	—	—	—	—	—	—
A/Nagasaki/HA-64/2009	27/11/2009	—	—	—	—	—	—	—	—	—	—	T	—	—	—	—	—	—	—	—	—	—	—	—
A/Nagasaki/HA-65/2009	3/12/2009	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
A/Nagasaki/HA-10-1/2010	4/1/2010	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
A/Nagasaki/HA-10-2/2010	4/1/2010	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
A/Nagasaki/HA-10-3/2010	4/1/2010	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
A/Nagasaki/HA-10-4/2010	4/1/2010	—	—	—	—	—	—	—	D	—	—	—	—	—	—	—	—	S	E	—	—	—	—	—
A/Nagasaki/HA-10-5/2010	12/1/2010	—	—	—	—	—	—	—	D	—	G	—	—	—	—	—	—	S	—	—	—	—	—	—
A/Nagasaki/HA-10-6/2010	12/1/2010	—	—	—	—	—	—	—	—	—	G	—	S	—	—	—	—	—	—	—	—	—	—	K
A/Nagasaki/HA-10-7/2010	12/1/2010	—	—	—	—	—	—	E	—	—	—	—	—	—	—	—	—	—	—	K	—	—	—	—
A/Nagasaki/HA-10-11/2010	12/1/2010	—	—	—	—	—	—	—	—	—	—	T	—	—	—	—	—	—	—	—	—	—	—	—
A/Nagasaki/HA-10-9/2010	18/1/2010	—	—	—	—	—	—	—	—	—	G	T	—	—	—	—	—	—	—	—	—	—	—	—
A/Nagasaki/HA-10-10/2010	18/1/2010	—	—	—	—	—	—	—	D	—	—	—	—	—	—	—	—	S	—	—	—	—	—	—
A/Nagasaki/HA-10-12/2010	18/1/2010	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	P	—	—
A/Nagasaki/HA-10-13/2010	18/1/2010	—	—	—	—	—	—	—	—	T	—	—	—	—	—	—	—	—	—	K	—	—	—	—
A/Nagasaki/HA-69/2010	20/1/2010	—	—	—	—	—	—	—	—	—	—	—	—	—	—	D	—	—	—	—	—	—	—	—
A/Nagasaki/HA-10-14/2010	1/2/2010	—	—	—	—	—	—	—	—	—	—	T	—	—	—	—	—	—	—	—	—	—	—	—
A/Nagasaki/HA-10-15/2010	1/2/2010	—	—	—	—	—	—	E	—	—	—	—	—	—	—	—	—	—	—	K	—	—	—	—
A/Nagasaki/HA-10-17/2010	8/2/2010	—	—	—	—	—	—	E	—	—	—	—	—	—	—	—	—	—	—	K	—	—	—	K
A/Nagasaki/HA-10-18/2010	15/2/2010	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	K	—	—	—	—
A/Nagasaki/HA-10-19/2010	15/2/2010	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
A/Nagasaki/HA-10-20/2010	15/2/2010	—	G	—	—	—	D	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
A/Nagasaki/HA-10-21/2010	22/2/2010	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	K	—	—	—	—
A/Nagasaki/HA-10-22/2010	1/3/2010	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
A/Nagasaki/HA-10-24/2010	8/3/2010	—	—	—	—	—	—	—	—	—	—	—	—	—	T	—	—	—	—	—	—	—	—	K
A/Nagasaki/HA-10-25/2010	8/3/2010	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	K	—	—	—	K
A/Nagasaki/HA-10-26/2010	15/3/2010	—	—	—	—	—	—	—	—	—	—	—	—	—	T	—	—	—	—	—	—	—	—	K
A/Nagasaki/HA-10-27/2010	15/3/2010	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	K
A/Nagasaki/HA-10-28/2010	23/3/2010	—	—	—	—	—	—	—	—	—	—	—	—	—	T	—	—	—	—	—	—	—	—	—
A/Nagasaki/HA-10-30/2010	29/3/2010	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	K

[1)]: Amino acid residues located in the Ca_2, Sb, and Ca_1 regions are indicated as (a), (b), and (c), respectively.

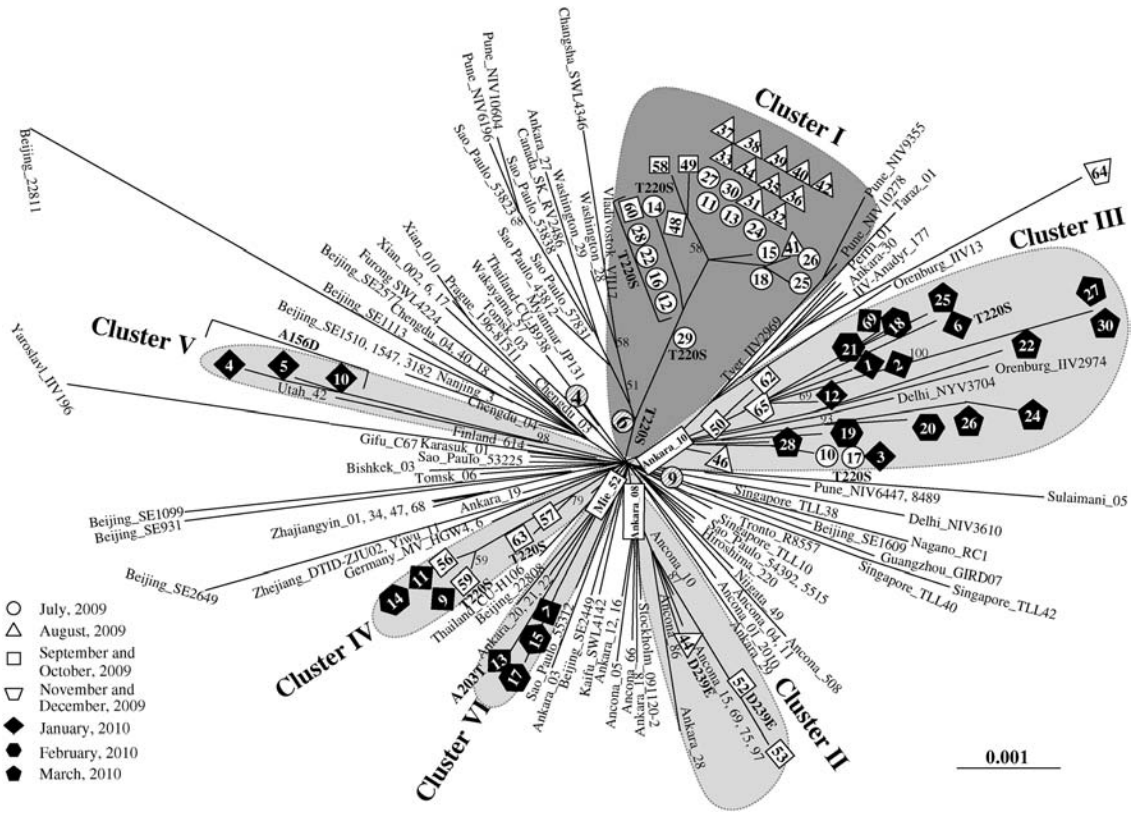

Fig. 1. Phylogenetic tree of the HA genes of the pandemic (H1N1) 2009 influenza viruses isolated in Nagasaki, Japan, between July 2009 and March 2010, determined by the maximum-likelihood method. Phylogenetic relationship of the HA sequences of Nagasaki isolates against those from global isolates collected between July 2009 and March 2010 was analyzed. Nagasaki clusters (I to VI) were indicated by ellipses and were highlighted. The amino acid mutations within antigenic sites of the Nagasaki isolates were plotted in the tree. The bar indicates one single nucleotide substitution per site. The bootstrap values that had a probability of >50% were shown in the tree.

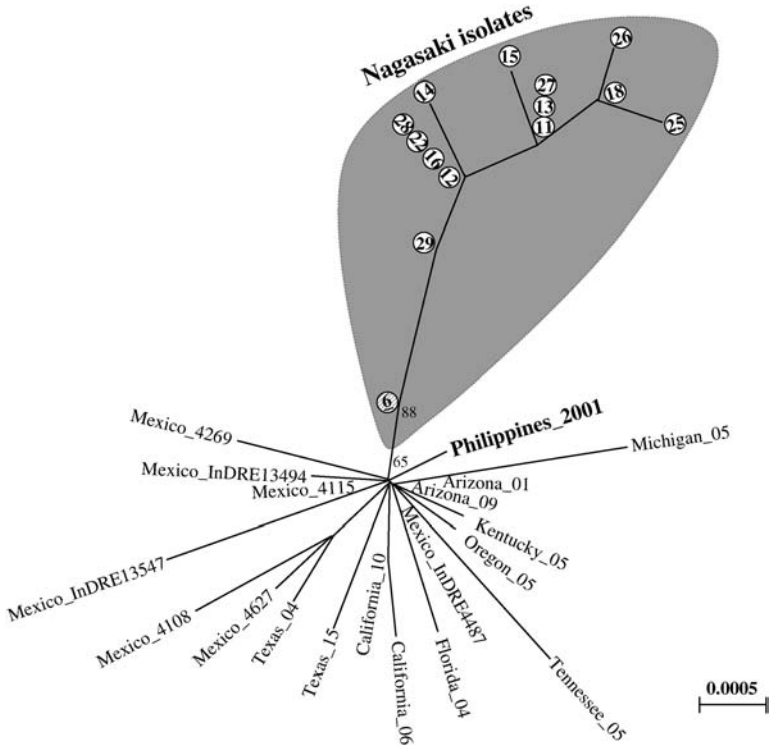

Fig. 2. Phylogenetic tree of the nucleotide HA sequences of the Nagasaki isolates classified as cluster I against those from A/Philippines/2001/2009, Mexican, and American isolates by the maximum-likelihood method. The bar indicates one single nucleotide substitution per site. The bootstrap values that had a probability of >50% were shown in the tree.

Table 3. Susceptibilities of Nagasaki isolates to oseltamivir in the NA inhibition assay

Strain name	H275Y mutation	Susceptibility to oseltamivir[1)]	
		IC_{50} (μM)	Fold change from HA-6
A/Nagasaki/HA-6/2009	−	1.8	
A/Nagasaki/HA-33/2009	−	2.0	1
A/Nagasaki/HA-58/2009	+	46	26

[1)]: The average IC_{50} values of duplicate measurements are shown. The fold increase in IC_{50} value compared to that of A/Nagasaki/HA-6/2009.

identical HA nucleotide sequences. Between the end of August 2009 and January 2010, 5 new clusters (II–VI) emerged, and 3 of these (clusters III, IV, and VI) continuously advanced until February or March 2010. Clusters II, III, and VI originated from A/Ankara/08/2009 (Turkey), A/Ankara/10/2009 (Turkey), and A/Mie/52/2009 (Japan), respectively. For strains in clusters IV and V, no original viruses were identified in the same cluster, but the viruses were more closely related to several other foreign (A/Ankara/10/2009) and Japanese (A/Mie/52/2009) isolates than other Nagasaki isolates in the different clusters. These results suggest that the onset of the pandemic influenza in Nagasaki originated from a patient who returned from the Philippines. Therefore, after September 2009, the spread of different strains of pandemic influenza viruses replaced the initially spreading virus.

To determine the evolutionary relationship to the original pandemic virus, we also performed phylogenetic analysis of the nucleotide HA sequences of Nagasaki isolates classified as cluster I against those from A/Philippines/2001/2009, Mexican, and American isolates. The nucleotide divergence increased from the initially transmitted viruses in the countries of the origin of the pandemic (Mexico and United States) to the Nagasaki isolates (Fig. 2). A/Nagasaki/HA-6/2009, isolated from a patient who returned from the Philippines (collected on July 13, 2009), was phylogenetically more similar to A/Philippines/2001/2009 (collected on May 20, 2009) than to the other Nagasaki isolates classified into Nagasaki cluster I.

Sensitivity of Nagasaki isolates to oseltamivir in an in vitro assay: To evaluate the oseltamivir resistance of A/Nagasaki/HA-58/2009, which possesses a H275Y mutation, we performed an in vitro oseltamivir-resistance assay. The 50% inhibitory concentration (IC_{50}) for A/Nagasaki/HA-58/2009 was 26-fold higher (46 μM) than that of A/Nagasaki/HA-6/2009 (1.8 μM), which does not have the H275Y mutation (Table 3). Similar sensitivity was observed between A/Nagasaki/HA-6/2009 and A/Nagasaki/HA-33/2009. These data clearly showed that A/Nagasaki/HA-58/2009 is significantly less sensitive to oseltamivir, most likely due to the H275Y mutation.

DISCUSSION

We herein report the characterization of the HA genes of the pandemic (H1N1) 2009 influenza viruses isolated in Nagasaki and their phylogenetic relationships with global isolates. The HA genes of 75 viruses collected in Nagasaki between July 2009 and March 2010 were analyzed. HA cleavage by a host protease is an important step in the replication cycle of the influenza virus and results in increased infectivity by activating the fusion potential (20,21). The HA of highly pathogenic avian strains contains a conserved arginine or a stretch of basic amino acids at the cleavage site (22). However, the predicted amino acid sequences of the HA of analyzed Nagasaki isolates did not contain such sequences. A similar observation in other pandemic (H1N1) 2009 strains was reported by Itoh et al. (23). The amino acid differences between the HA of Nagasaki isolates and global isolates are summarized in Table 2. By employing 3D structures to map amino acid residues in the antigenic sites of HA, Igarashi et al. showed that the HA1 antigenic structure of the pandemic (H1N1) 2009 virus (A/California/04/2009) is similar to that of the 1918 (H1N1) pandemic virus (24). Similarly, Itoh et al. showed that appreciable neutralizing antibodies against the A/California/04/2009 strain were present in the sera collected from individuals born before 1918 (23). We further compared the amino acid residues constituting the 4 antigenic sites of A/California/04/2009 with those of the Nagasaki isolates. Almost all residues were highly conserved in the Nagasaki isolates (data not shown), indicating that HA proteins from the isolated viruses have an antigenic structure similar to that of the 1918 (H1N1) pandemic virus. It is worth noting that several amino acid differences in the antigenic sites of Ca_1 (T220S in A/Nagasaki/HA-6, 12, 14, 16, 17, 22, 28, 29, 59, 60, 63/2009, and A/Nagasaki/HA-10-6/2010), Ca_2 (A156D in A/Nagasaki/HA-10-4, 5, and 10/2010 and D239E in A/Nagasaki/HA-44 and 52/2009), and Sb (A203T in A/Nagasaki/HA-10-13/2010 and D204G in A/Nagasaki/HA-10-5, 6, and 9/2010) were also observed. Our data suggest that an antigenic drift may have occurred during the spread within the human population, which potentially lowered the efficacy of pandemic influenza vaccines. To observe the pattern of spreading of the amino acid mutations within HA antigenic sites, we plotted these mutations in the phylogenetic tree (Fig. 1). Interestingly, T220S (located within Ca_1) in cluster I seems to have disappeared starting in August (except for A/Nagasaki/HA-60/2009), and this mutation sporadically reemerged in clusters III and IV. On the other hand, A156D (located within Ca_2) in cluster V is likely to have selectively emerged as a cluster V virus. These findings will be useful as a reference for future studies for estimating the mechanism of antigenic drift events.

The results of the phylogenetic analysis revealed that the Nagasaki isolates could be classified into 6 main clusters (I–VI) (Fig. 1). Almost all Nagasaki isolates collected in the early season were classified into cluster I, which most probably evolved from A/Nagasaki/HA-6/2009 isolated from a patient who returned from the Philippines. To confirm this, we further compared the nucleotide sequences of HA of A/Nagasaki/HA-6/2009 with those from strains identified in the Philippines. The nucleotide sequences of HA from the A/Nagasaki/HA-6/2009 strain showed a significantly high similarity with that of the A/Philippines/2001/2009 isolate (collected on May 20, 2009) (Fig. 2). These results

raise the possibility that patient HA-6 had been infected by the virus that had originated in the Philippines. The Philippine isolate also showed higher similarities with those identified in Mexico and the United States than the Nagasaki isolates, and this isolate most likely originated from the countries in which the pandemic started. The reason why the A/Nagasaki/HA-4/2009 and A/Nagasaki/HA-9/2009 strains, apparently ''imported'' from foreign countries, did not spread might be because of the rapid isolation and subsequent treatment of the affected patients.

Sixteen strains with identical HA sequences (A/Nagasaki/HA-11, 13, 24, 27, 30–40, and 42/2009) were classified into cluster I. Among these strains, A/Nagasaki/HA-13/2009 was isolated from a patient who had symptoms after participating in the summer festival held in Nagasaki (Table 1). Furthermore, 3 strains (A/Nagasaki/HA-12, 14, 15/2009), isolated from participants in the same festival, also appeared in cluster I. Approximately 2,000 people from Nagasaki as well as from outside the city participated in the festival. This suggested that the initial spread of the pandemic (H1N1) 2009 influenza viruses occurred among those who participated in the event.

From the end of August, 5 new phylogenetic clusters appeared, and 3 of these (III, IV, and VI) continuously spread until February (IV and VI) or March 2010 (III). Clusters II, III, and VI originated from A/Ankara/08/2009 (Turkey), A/Ankara/10/2009 (Turkey), and A/Mie/52/2009 (Japan), respectively, as the estimated original viruses. Strains in clusters IV and V without a suspected origin were evolutionarily more closely related to several other foreign (A/Ankara/10/2009) and Japanese (A/Mie/52/2009) isolates than to other Nagasaki isolates in the different clusters (Fig. 1). These results imply that the mid- to late-season of the Nagasaki pandemic may have resulted from different isolates that originated outside of Nagasaki. Through this study, we succeeded in simulating how pandemic influenza viruses spread in a region far from the pandemic origin. In several countries, quarantine strategies tended to be thought of as relatively ineffective. However, on the basis of the data from this study, such strategy is likely to be effective in the case of a small city such as Nagasaki. Our findings emphasize the importance of quick tests at airports or seaports and the use of quarantine in preventing the spreading of influenza viruses.

In the present study, we also analyzed the NA sequence of the isolated viruses. We detected one H275Y mutant isolated from patient HA-58 who had aggravated symptoms despite treatment with oseltamivir in October 2009 (Table 1). The mutant strain was less sensitive to oseltamivir compared with other Nagasaki isolates without the H275Y mutation in the NA sequence as shown in an in vitro oseltamivir-resistance assay (Table 3). In the past, the H275Y mutant had been recognized as being of relatively low clinical consequence due to reduction of the NA activity required for viral replication (25). However, the spread of oseltamivir-resistant influenza viruses was very rapid, because more than 90% of the seasonal H1N1 influenza viruses isolated during the 2008/2009 season were oseltamivir resistant (14). In our present study, the other 74 Nagasaki isolates were not found to be H275Y mutants. Therefore, it is important to pay close attention to the emergence of oseltamivir-resistant viruses.

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Conflict of interest None to declare.

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