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**Short Communication**

# Phylogenetic Analysis of an Off-Seasonal Influenza Virus A (H3N2) in Niigata, Japan, 2010

Clyde Dapat*, Yasushi Suzuki, Miyako Kon[1], Tsutomu Tamura[1], Reiko Saito, Isolde C. Dapat, Osamu Yamazaki[2], Takato Odagiri[3], Seiichiro Fujisaki[3], and Hiroshi Suzuki[4]

*Department of Public Health, Graduate School of Medical and Dental Sciences, Niigata University, Niigata 951-8510;*
*[1]Virology Section, Niigata Prefectural Institute of Public Health and Environmental Sciences, Niigata 950-2144;*
*[2]Department of Health and Social Welfare, Niigata Prefectural Government, Niigata 950-8570;*
*[3]Influenza Virus Research Center, National Institute of Infectious Diseases, Tokyo 208-0011; and*
*[4]Department of Nursing, Niigata Seiryo University, Niigata 951-8121, Japan*

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**SUMMARY**: The objective of this study was to characterize the off-seasonal influenza virus A subtype H3N2, which caused an outbreak in an elderly hospital in Niigata, Japan. Virus isolates were subtyped by the hemagglutination-inhibition test and screened for antiviral drug sensitivity by real-time PCR using cycling probe technology and the 50% inhibitory concentration ($IC_{50}$) method. Whole genome sequencing was performed in order to determine the phylogeny of the outbreak virus. Seven virus isolates were analyzed in this study, and the results showed that all belonged to the influenza virus A (H3N2). These viruses exhibited the S31N mutation in M2, which confers resistance to amantadine. The results of the $IC_{50}$ analysis showed that these viruses were sensitive to both oseltamivir and zanamivir. Whole genome analysis revealed that the virus was similar to the A/Perth/16/2009 strain and that it is a triple reassortant virus with a 3 + 3 + 2 pattern of segment recombination.

Influenza outbreaks are common among residents in elderly hospitals and long-term care facilities (1). To contain the outbreak, antiviral drugs are administered to residents and staff (2). M2 ion channel blockers (amantadine and rimantadine) and neuraminidase inhibitors (NAIs) (oseltamivir and zanamivir) are the two classes of drugs used for combating influenza. These antivirals are indispensible during an outbreak, especially when a mismatch occurs between the vaccine and the circulating strain (3,4). In the 2009–2010 season, the pandemic (H1N1) influenza A virus and subtype H3N2 virus were resistant to amantadine (5). Influenza outbreaks usually occur during the winter season in temperate countries. We report an off-seasonal influenza outbreak in an elderly hospital in Niigata, Japan, in July 2010, wherein the causative agent was the amantadine-resistant H3N2 virus. We performed antiviral susceptibility assay and whole genome analysis to characterize this virus.

During the summer month of July in 2010, an influenza outbreak was reported by the elderly hospital to the prefectural public health office. The hospital houses residents with mental conditions, such as senile dementia. Patients with influenza-like symptoms, such as fever and cough, were tested using rapid test kits. The prefectural office is generally blinded to the identity of the manufacturer of the rapid test kit; therefore we cannot report it here. Nasopharyngeal swabs exhibiting positive results in the rapid test were sent to the Division of Virology, Niigata Prefectural Institute of Public Health and Environmental Sciences for virological examination. The collected samples were then inoculated into MDCK cells. Virus isolates were characterized by the hemagglutination-inhibition (HI) test and subtyped by real-time PCR assay (6).

Analyses of antiviral drug resistance and whole genome sequencing were performed at the Department of Public Health, Niigata University. Screening for the S31N mutation in M2, which confers amantadine resistance, was performed by real-time PCR using the cycling probe technology (7). Screening for NAI susceptibility was performed by the 50% inhibitory concentration ($IC_{50}$) method with oseltamivir carboxylate (F. Hoffman-La Roche, Basel, Switzerland) and zanamivir (GlaxoSmithKline Research and Development, Hertfordshire, UK) (8,9).

Whole genome sequencing was performed using a Big Dye Terminator v3.1 cycle sequencing kit, and the sequencing products were resolved using ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, Calif., USA) (10). Sequence contigs were assembled using the Lasergene SeqMan Pro package, version 7.2.1 (DNAStar, Madison, Wis., USA), and the sequences were edited using the BioEdit software (http://www.mbio.ncsu.edu/BioEdit/). Phylogenetic analysis was performed using the MEGA 4.0 software (11). Nucleotide sequences used in this study have been deposited at GenBank (accession nos. HQ703347–HQ703402).

The outbreak occurred during the summer month of July 2010 when the incidence of the pandemic (H1N1)

*Corresponding author: Mailing address: Department of Public Health, Graduate School of Medical and Dental Sciences, Niigata University, 1-757 Asahimachi-dori, Niigata City, Niigata 951-8510, Japan. E-mail: clyde@med.niigata-u.ac.jp

2009 infection was low and H3N2 virus infection was sporadic in Japan (12). Of the 59 residents, 32 (54%) were found to be infected with the influenza virus on the basis of the results of the rapid test. Of the 30 healthcare workers, 8 had fever but only 1 was tested with the rapid test kit, and the result was negative. The mean age of the residents who were infected with the virus was 85 years old. A total of 10 nasopharyngeal swabs from the residents were received for virological examination at the Division of Virology, Niigata Prefectural Institute of Public Health and Environmental Sciences. All 10 samples were positive for influenza virus A (H3N2) by real-time PCR assay. Of the 10 samples, 9 were positive for virus isolation. The results of the HI test revealed that the H3N2 virus was antigenically related to A/Victoria/210/2009, which was similar to the A/Perth/16/2009 strain (Table 1).

Table 1. Hemagglutination-inhibition test of influenza virus A (H3N2) in Niigata, Japan in 2010

| | Postinfection sera | |
|---|---|---|
| Antigens | A/Victoria/210/2009 | A/Uruguay/716/2007 |
| A/Victoria/210/2009[1)] | 640 | nd[3)] |
| A/Uruguay/716/2007[2)] | nd[3)] | 320 |
| Isolate | | |
| A/Niigata/1144/2010 | 640 | 10 |

[1)]: A/Perth/16/2009-like.
[2)]: A/Brisbane/10/2007-like.
[3)]: not determined.

Virus isolates were forwarded to the Department of Public Health, Niigata University for molecular characterization. Real-time PCR assay using the cycling probe technology showed that all samples exhibited the S31N mutation in M2, which confers resistance to amantadine. This was confirmed by M2 gene sequencing. The results of the NAI assay showed that the mean $IC_{50}$ values of the H3N2 virus were 1.32 ± 0.48 nM and 1.07 ± 0.60 nM for oseltamivir and zanamivir, respectively; these values were within the range observed in viruses sensitive to these antiviral preparations. This indicates that the outbreak viruses were susceptible to both NAIs. Oseltamivir was administered to residents who had positive results in the rapid test.

Whole genome sequencing was performed on 7 outbreak viruses. Phylogenetic analyses showed that these viruses were identical to each other and formed a single

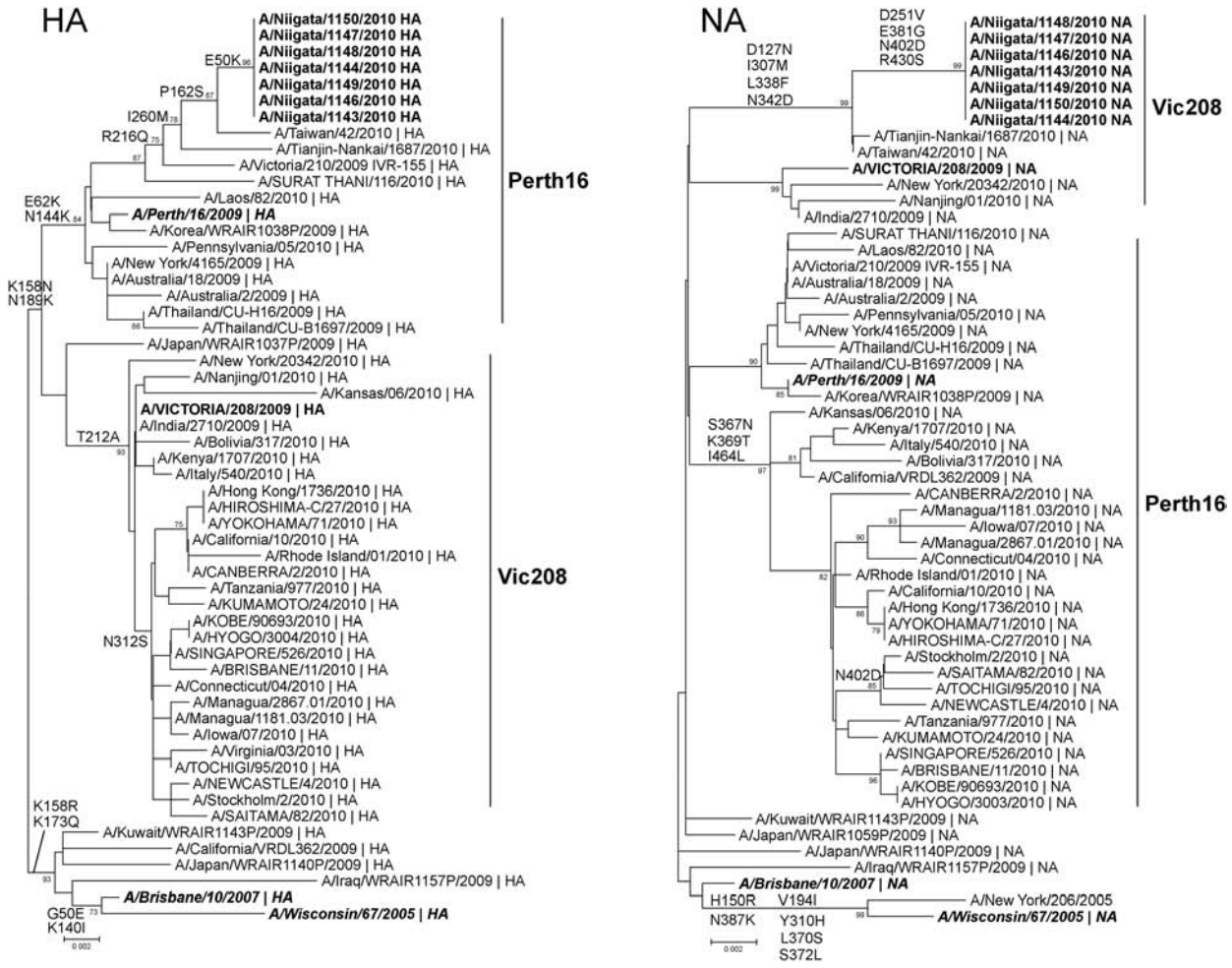


Fig. 1. Phylogenetic analysis of the HA and NA genes of influenza virus A (H3N2) isolates in Niigata, Japan in 2010. Trees were inferred using neighbor-joining method with bootstrap values of 1,000 replicates and amino acid changes are shown on the left side of the node. Isolates used in this study are boldfaced. Vaccine strains are italicized and boldfaced. Perth16 indicates A/Perth/16/2009-like while Vic208 refers to A/Victoria/208/2009-like group. Scale bar indicates nucleotide substitutions per site.

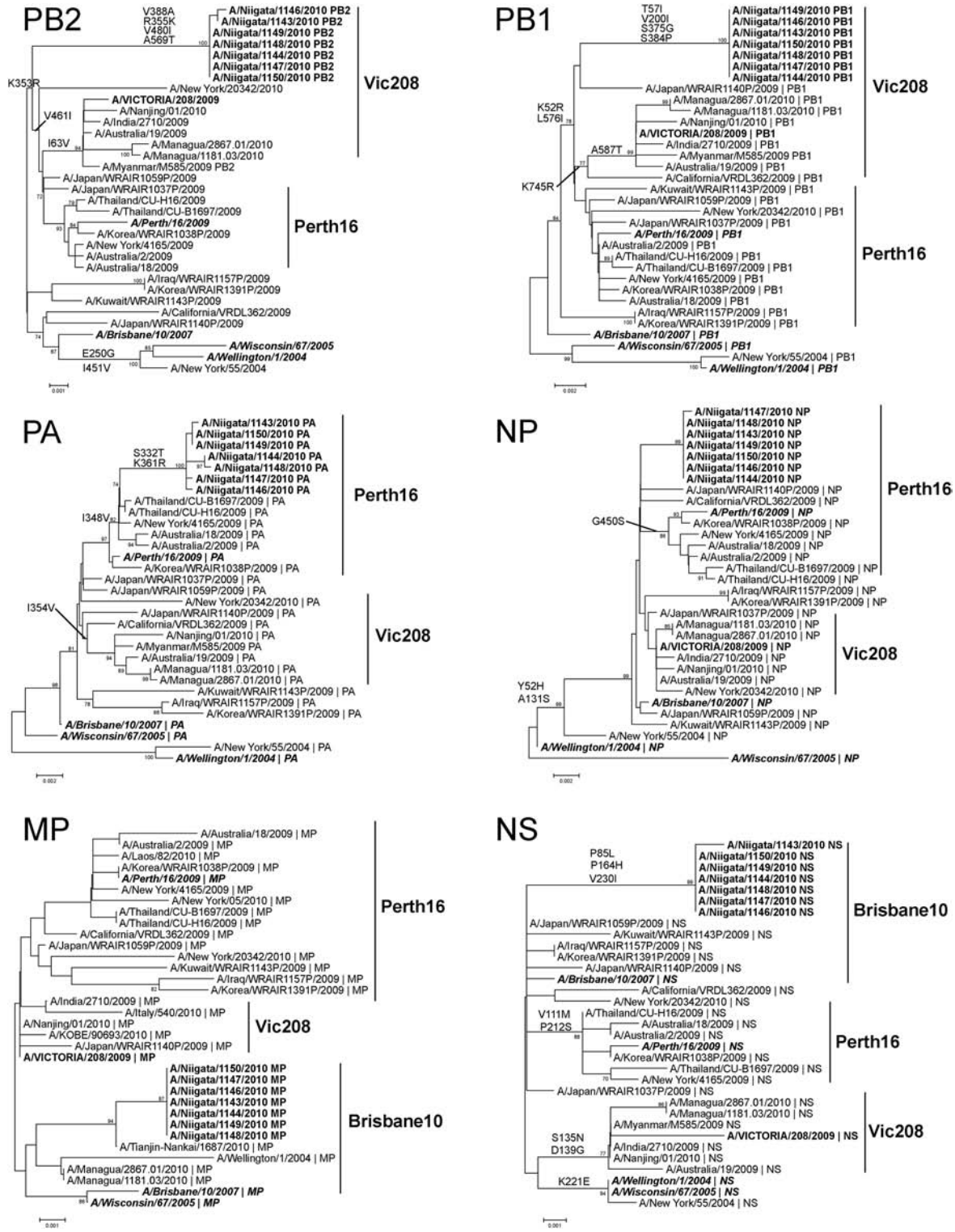


Fig. 2. Phylogenetic analysis of the PB2, PB1, PA, NP, MP, and NS genes of influenza virus A (H3N2) isolates in Niigata, Japan in 2010. Trees were constructed using neighbor-joining method. Figures at the nodes indicate confidence levels of bootstrap analysis using 1,000 replicates as percentage value. Amino acid mutations that characterize a branch are indicated on the left side of the node. Isolates used in this study are boldfaced. Vaccine strains are italicized and boldfaced. Perth16 indicates A/Perth/16/2009-like while Vic208 refers to A/Victoria/208/2009-like group. Scale bar indicates nucleotide substitutions per site.

cluster. Analysis of the HA gene showed that the virus was genetically related to the A/Perth/16/2009 strain (Fig. 1). Five amino acid mutations in HA were detected at the antigenic sites B (P162S), C (E50K), D (S214I), and E (I260M and R261Q) (13). This suggests that the virus was a genetic variant of the A/Perth/16/2009 strain. Sequence analyses of the other gene segments indicated that the outbreak H3N2 virus was a reassortant. Polymerase acidic protein (PA) and nucleocapsid protein (NP) phylogenies revealed that it was similar to the

Table 2. Amino acid substitutions in subtype H3N2 outbreak viruses compared with A/Perth/16/2009 vaccine strain

| PB2 | PB1 | PA | HA | NP | NA | M1 | M2 | NS1 | NS2 |
|---|---|---|---|---|---|---|---|---|---|
| V338A | T57I | S332T | E50K | —[1)] | D127N | —[1)] | —[1)] | P85L | — |
| R355K | V200I | I348V | P162S | | D251V | | | P164H | |
| V480K | S375G | K361R | S214I | | I307M | | | V230I | |
| A569T | S384P | | I260M | | L338F | | | | |
| | | | R261Q | | N342D | | | | |
| | | | | | E381G | | | | |
| | | | | | N402D | | | | |
| | | | | | R430S | | | | |

1): — indicates no amino acid changes.

A/Perth/16/2009 strain (Fig. 2). However, analyses of neuraminidase (NA), polymerase basic protein 2 (PB2), and polymerase basic protein 1 (PB1) segments showed that the virus was related to the A/Victoria/208/2009 strain. The matrix protein (MP) and nonstructural protein (NS) phylogenies revealed that the virus was genetically similar to an older strain, A/Brisbane/10/2007 (Fig. 2). When compared with the vaccine strain, A/Perth/16/2009, the outbreak virus exhibited 27 amino acid changes, with 5 substitutions in HA, 8 in NA, 4 in PB2, 4 in PB1, 3 in PA, and 3 in NS1 (Table 2).

The virus responsible for the outbreak probably emerged as a result of a 3 + 3 + 2 segment reassortment event involving the 3 abovementioned strains. The emergence of this reassortant virus may be attributed to the cocirculation of the phylogenetically distinct but antigenically similar stains A/Perth/16/2009 and A/Victoria/208/2009 during the previous influenza season (5). In 2010, these 2 strains continued to cocirculate in Japan. The A/Victoria/208/2009-like strains were collected in spring (April), and the A/Perth/16/2009-like viruses were detected in summer (July) (12).

The emergence of new variants of the influenza virus may be the result of different evolutionary mechanisms, such as random point mutation and genetic reassortment (14,15). Mutations in the viral genome can be attributed to the high error rate of the viral RNA polymerase as well as selection pressure from the host's immune system, while genetic reassortment possibly occurs because of the segmented nature of the viral genome (16). In our previous study with earlier strains of amantadine-resistant H3N2 viruses, we observed reassortant viruses with gene segments from 3 independent strains (17). Similarly, in the present study, we detected reassortant viruses, in addition to random point mutations in the genomes of H3N2 strains. These findings imply that reassortment plays a major role in the evolution of influenza virus A (H3N2). This study highlights the importance of analyzing the entire genome for characterizing novel strains. Deposition of all analyzed whole genome sequences in public databases is warranted because this will facilitate the elucidation of the genetic background and evolution of these viruses.

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**Conflict of interest** None to declare.

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