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Low Hemagglutinin-Titer Strains of Pandemic Influenza A (H1N1) 2009 Virus Circulated in Toyama Prefecture, Japan, during the 2009–2011 Influenza Seasons

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The pandemic influenza A (H1N1) 2009 [A(H1N1)pdm09] virus continues to circulate worldwide. During the 2010–2011 season, however, virus circulation in temperate northern hemisphere countries occurred with a seasonal influenza pattern (1). In Toyama Prefecture, Japan, the influenza epidemic in the 2010–2011 season was slightly greater than that which had been seen in previous seasons. Influenza A(H1N1)pdm09 virus predominated over influenza A (H3N2) and type B viruses throughout Japan, including Toyama Prefecture over the past two influenza seasons (2). A total of 124 A(H1N1)pdm09 virus isolates were obtained at the Toyama Institute of Health during the 2010–2011 season. During this season, we had difficulty of performing hemagglutination inhibition (HI) assays for antigenic characterization of the A(H1N1)pdm09 virus isolates, because of their low hemagglutinin (HA) titers. That is, to be subjected to an HI assay, standardized antigens must have an HA titer of 8 HA units per 50 μl (hereafter, 8 HA). Although these isolates induced clear cytopathic effects on infected Madin-Darby canine kidney (MDCK) cells, the majority of them showed low HA titers (less than 8 HA), as assayed using guinea pig erythrocytes. This was thought to be due to changes in receptor binding characteristics of HA, such as the loss of agglutination of chicken erythrocytes by A (H3N2) and A (H1N1) viruses that was observed in the early 1990s (3,4). We therefore characterized the low HA titer isolates of influenza A(H1N1)pdm09 virus that were obtained from clinical specimens during the 2009–2010 and 2010–2011 influenza seasons in Toyama Prefecture, Japan.

First, we compared the HA titers of influenza A(H1N1)pdm09 virus isolated during 2009–2010 and 2010–2011 seasons (Fig. 1). For the 2009–2010 season, 306 virus isolates were identified as influenza A(H1N1)pdm09 by real-time reverse transcriptase-polymerase chain reaction, following a procedure described previously (5). Of these, 88 were selected randomly for the HA assay, and 61 (69%) showed more than 4 HA with 0.75% guinea pig erythrocytes. In contrast, 95 of 121 (79%) isolates from the 2010–2011 season showed less than 8 HA, indicating that HA titers of the culture supernatant of MDCK cells after replication of the majority of A(H1N1)pdm09 virus isolates in this season were low and insufficient to be subjected to HI assay. Additionally, these isolates were further assayed against chicken, turkey, and goose erythrocytes in a 0.5% suspension. The HA titers obtained using turkey and goose erythrocytes were similar to that obtained using guinea pig erythrocytes (data not shown). Chicken erythrocytes gave a lower HA titer than the erythrocytes

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We analyzed the HA and neuraminidase (NA) genes because it was hypothesized that the low HA titer was due to reduced affinity of HA for erythrocytes. Each of 10 isolates with low or high (≥8 HA) HA titers obtained in the 2009–2010 and 2010–2011 seasons was subjected to nucleotide sequencing. The HA and NA gene-specific primers established by the National Institute of Infectious Diseases, Japan were used in this study. The nucleotide sequences have been submitted to the DNA Data Bank of Japan (accession nos. AB649856–AB649935). The phylogenetic tree of the HA1 region is shown in Fig. 2A. With the exception of A/Toyama/92/2010, all isolates with HA titers lower than 4 HA (bold font) in the 2009–2010 (regular font) and 2010–2011 (italicized font) seasons belonged to the same clade, with a common characteristic amino acid substitution from alanine to threonine at position 197 (A197T). Additionally, the isolates for the 2010–2011 season formed a subclade represented by an amino acid substitution from serine to threonine at position 185. In contrast, the phylogenetic tree of the NA gene showed that the low HA titer isolates, with the exception of A/Toyama/92/2010 were divided into two clades (Fig. 2B). The 2009–2010 seasonal isolates belonged to a clade harboring a substitution from aspartic acid to asparagine at position 416. The 2010–2011 seasonal isolates belonged to another clade harboring substitutions from asparagine to serine at position 44 and from leu-
cine to methionine at position 415. These NA mutations were located far from the active site of the NA protein (data not shown), suggesting that the low HA titer was unlikely to be caused by increased NA activity. Collectively, these results raise the possibility that the A197T substitution in the HA protein is critical for the reduced affinity of HA for erythrocytes.

To clarify the three-dimensional (3-D) location of the A197T substitution in the HA protein, we constructed a 3-D model of the HA1 trimer of A/Toyama/26/2011, which is a representative strain of low HA titer by the homology modeling technique (6,7). The model showed that the A197T substitution occurred near the 190-helix of HA1 (Fig. 3). Because this helix participates in the binding to the infection receptor (7), it is possible that the A197T substitution indirectly alters the receptor binding properties of the HA by influencing the orientation of side chains of the 190-helix. Further study of the interaction between the HA protein and receptor on the erythrocytes will be needed to address this issue.

Our finding raises the question of whether the A(H1N1)pdm09 virus that possesses the A197T substitution is also circulating worldwide. To address this question, we searched for the HA gene of the A(H1N1)pdm09 virus in the Global Initiative on Sharing All Influenza Data EpiFlu database (http://platform.gisaid.org/dante-cms/struktur.jdante?aid=1131). A total of 857 clinical isolates, collected between September 1, 2010 and July 20, 2011, had been submitted to the database by the World Health Organization (WHO) Collaborating Centers for influenza (as of July 20, 2011). Among these, 164 isolates possess the A197T substitution [Africa, 0 of 39 (0%); Asia, excluding Japan, 22 of 57 (39%); Europe, 22 of 375 (6%); Japan excluding Toyama, 92 of 131 (70%); North America, 23 of 186 (12%); Oceania, 5 of 61 (8%); and South America, 0 of 8 (0%)]. The data show that the proportion of the A197T mutant virus circulating in the global community is substantially larger in Asia, particularly in Japan, than in the other regions. It is still unclear as to whether the foreign isolates also give low HA titers and whether the A197T substitution contributes to the viral fitness of the A(H1N1)pdm09 virus. Additionally, we cannot rule out the possibility that the A197T mutants grow poorly in tissue culture cells or that the HA activity of those viruses is unstable. A detailed analysis of the virus possessing this particular amino acid substitution remains to be carried out.

Although the HI assay remains the gold standard test within the WHO Global Influenza Surveillance Network for determining antigenic characteristics of influenza virus isolates and influenza vaccine strains (8), a neutralization assay might be required for the isolates presented in the current study. The data presented here could be useful for the continued surveillance of virus strains in the coming seasons, both for monitoring the spread and determining the epidemiological impact of the influenza A(H1N1)pdm09 virus strain possessing the A197T substitution in the HA protein.

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Conflict of interest None to declare.

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