Frequent Isolations of Influenza A Viruses (H1N1)pdm09 with Identical Hemagglutinin Sequences for More Than Three Months in Japan

Yu Yoshida,*† Akeno Tsuneki,* Asao Itagaki,* Hideaki Tsuchie,‡§ Takayoshi Okada,‡‖ Sakae Narai,‡‖ Masaaki Kasagi,‡¶ Kiyoshi Tanaka,‡ Akiko Ito,*† Kazuo Ryoke† and Seiji Kageyama*‡**

**Division of Virology, Department of Microbiology and Immunology, School of Medicine, Tottori University Faculty of Medicine, Yonago 683-8503, Japan, †Division of Oral and Maxillofacial Biopathological Surgery, Department of Medicine of Sensory and Motor Organs, School of Medicine, Tottori University Faculty of Medicine, Yonago 683-8503, Japan, ‡Tottori Infectious Diseases Forum, Tottori Prefecture, Japan, §Tsuchie Internal Medicine and Pediatric Clinic, Sakaiminato 684-0011, Japan, ‖Department of Pediatrics, Tottori Prefectural Kousei Hospital, Kurayoshi 682-0804, Japan, ¶Kasagi Children's Clinic for Health Service, Yonago 683-0822, Japan and **Tanaka Pediatric Clinic, Tottori 680-0844, Japan*

ABSTRACT

Background Although it has been suggested that antigenic drift does not occur in a single epidemic season in temperate countries, there is not enough evidence on the circulation period of influenza virus with identical nucleotide sequences. Therefore, strains of influenza virus were isolated sequentially during five consecutive epidemic seasons in Japan and their nucleotide sequences were determined.

Methods Nasal swabs or aspirated nasal discharges were collected from influenza A virus antigen-positive individuals living in Tottori Prefecture, Japan for five consecutive winters starting in 2009–2010, and subjected to viral isolation, determination of hemagglutinin nucleotide sequence and phylogenic analyses. The nucleotide sequences were compared with each other and also with those of foreign strains in the International Nucleotide Sequence Database.

Results Totally 288 A(H1N1)pdm09 strains were tested and those composed 38 clusters with identical ones displaying 100% nucleotide homology. One strain showed sequential infections more than three months without any detectable mutation, and a maximum interval of two detection timings of strains was 94 days. This implies that influenza viruses mutate rarely in an epidemic season in Japan if they can be hypothesized, mutation frequency of influenza viruses being mostly the same among strains. Among these identical strains, two strains were not only identical to other Japanese isolates, but also to those isolated in Mongolia and Thailand in the same epidemic season.

Conclusion These results suggest that genetic drift has occurred infrequently in Japan as shown in some other countries. The drifted strains may have generated somewhere else and entered into Japan. These results support the proposed 'sink-source' model of viral ecology in which new lineages are seeded from a persistent influenza reservoir in tropical countries to 'sink' populations in temperate regions including Japan.

Key words genetic drift; influenza A virus; Japan; sequence homology; temperate country

An international influenza study group estimated the global burden of respiratory infections due to seasonal influenza in young children in 2008. The findings revealed that 90 million new cases of influenza, 20 million cases of influenza-associated acute lower respiratory infections, and one million cases with severe symptoms, occurred worldwide in children younger than 5 years.[1] Older adults (over 65 years) are also vulnerable to influenza, with the highest prevalence mainly occurring in this age group.[2] Increasing age has been associated with a higher risk of hospitalization due to influenza during an influenza epidemic season.[3, 4]

The human immune response to viral infection is not completely cross-protective. Therefore, natural selection favors amino acid variants of the hemagglutinin and neuraminidase proteins that allow the virus to evade immunity. The continual change observed in the antigenic structure over time is referred to as antigenic drift. However, this process does not appear to occur within the time frame of a single epidemic season in a single locality and few amino acid changes occur in hemagglutinin within a local population at the seasonal scale.[5] Eight identical viruses identified in a French study and these virus groups appeared to have clustered due to the accumulation of secondary infections in this locality. The average nucleotide diversity was 0.0034 nucleotides per site in this study.[6] Another study concluded that no major clade of viruses in a season appeared to have evolved in New York State and a virus circulating in this area was mostly replenished each season from an extensive global gene pool.[7] Although it has been estimated that antigenic drift does not occur in a single epidemic

Corresponding author: Seiji Kageyama, MD, PhD
skageyama@med.tottori-u.ac.jp
Received 2015 October 7
Accepted 2015 October 21
Abbreviations: MDCK, Madin-Darby canine kidney; MEGA 6, Molecular Evolutionary Genetics Analysis Version 6.0; PCR, polymerase chain reaction; RT, reverse transcribed

season as described above, further research is needed to address the time scale on which this antigenic process occurs, as suggested previously.[5]

In the present study, the mutational profile of the seasonal influenza A viruses (H1N1)pdm09 that spread among community members in a local setting in Japan was investigated over an interval during which a pair of identical strains could be isolated.

MATERIALS AND METHODS

Samples

After written informed consent was obtained, nasal swabs and/or aspirated nasal discharges were collected from influenza A virus antigen-positive and suspected patients living in Tottori Prefecture, Japan, during five consecutive winters since 2009-2010 (Table 1). Each swab or aspirate was kept in 3 mL of a virus transportation medium [2% Difco Veal Infusion Broth (Becton Dickinson, Tokyo, Japan), 0.4% gelatin, 0.06% bovine serum albumin, 200 unit/mL penicillin G, and 100 μg/mL streptomycin in Dulbecco's Modified Eagle Medium (Thermo Fisher Scientific, Waltham, MA)] at 4 °C prior to virus isolation. The Institutional Review Board of Tottori University Faculty of Medicine approved this study protocol.

Virus isolation

The procedure for influenza virus isolation was based on the manual issued by the World Health Organization with slight modifications.[8] Briefly, an aliquot (500 μL) of the diluted sample in transportation medium was inoculated into the Madin-Darby canine kidney (MDCK) cell line for one hour under a 5% CO_2/95% air atmosphere. The culture was maintained in Dulbecco's Modified Eagle Medium supplemented with 10 μg/mL trypsin (Difco Trypsin 250, Becton Dickinson), 0.2% heat-inactivated bovine serum albumin, 4 mM L-glutamine, 200 units/mL penicillin G, and 100 μg/mL streptomycin at 34 °C under a 5% CO_2/95% air atmosphere until the recognition of cytopathic effects. Culture medium was kept frozen at –80 °C as a virus stock prior to further studies.

RNA extraction, Reverse Transcription, and Polymerase Chain Reaction

Total RNA was extracted using SMITEST EX-R&D (MBL, Nagoya, Japan) and QIAamp Viral RNA Mini Kit (Qiagen, Tokyo, Japan). Eight RNA segments of influenza A virus were simultaneously reverse-transcribed (RT) and amplified using the SuperScript III One Step RT-Polymerase Chain Reaction (PCR) System (Life Technologies, Tokyo, Japan) with the MBTuni-12/MBTuni-13 primer pair.[9]

The first round of PCR products were subjected to a second round in order to amplify A(H1N1)pdm09 (266 bases) for sub-typing.[10] Primers uploaded to the World Health Organization website 'Pandemic H1N1 2009 guidance documents' and others were used for the amplification of a longer hemagglutinin segment, A(H1N1)pdm09.[11] These second PCR products were separated on 2% agarose gel, stained with ethidium bromide, and visualized under ultraviolet light.

Analyses of nucleotide sequences and diversity

The nucleotide sequences of the amplified hemagglutinin fragments were determined using BigDye Terminators v3.1 Cycle Sequence kit according to the manufacturer's instructions (Life Technologies). M13 and PCR primers were used for the sequencing reaction. Nucleotide sequences except primer regions [1,701 bases of A(H1N1)pdm09] were aligned with those retrieved from the International Nucleotide Sequence Database. Inspection and manual modification and evolutionary analyses were conducted in Molecular Evolutionary Genetics Analysis Version 6.0 (MEGA 6).[12] A phylogenetic tree was constructed by neighbor-joining method (1,000 bootstrap replications) in MEGA 6. An estimation of the mean evolutionary diversity was also conducted using MEGA 6.

Table 1. Numbers of tested samples and clusters of identical strains, and diversity of strains per year

Season*	A(H1N1)pdm09			A(H3N2)			B		
	N†	Clusters‡	Diversity§	N	Clusters	Diversity	N	Clusters	Diversity
2009-2010	144	22	0.0040	0	0	nd	0	0	nd
2010-2011	144	16	0.0048	25	2	0.0052	31	7	0.0078
2011-2012	0	0	nd	0	0	nd	3	1	0.0000
2012-2013	0	0	nd	0	0	nd	6	1	0.4411
2013-2014	0	0	nd	0	0	nd	20	6	0.0356
Total	288	38		25	2		60	15	

*From July 1 to June 30 of the following year. †The number of tested samples with nucleotide sequences that could be determined. ‡Composed of identical strains. §Numbers of base substitutions per site from mean diversity calculations for the entire population are shown. nd: not done.

Accession numbers

The sequences described in this study have been deposited in the International Nucleotide Sequence Database under accession numbers AB745121–AB745408.

RESULTS

Phylogenetic analysis of influenza A virus (H1N1) pdm09 in five consecutive seasons in Japan

Twenty-two clusters (no. 17–38) of isolates in 2009–2010 and sixteen (no. 1–16) in 2010–2011 were composed of identical strains displaying 100% homology within the nucleotide sequences of A(H1N1)pdm09 hemagglutinin. (Table 1 and Fig. 1). The limited number of influenza A(H3N2) and B strains were also analyzed and are shown in Table 1. Clusters composed of strains with identical hemagglutinin nucleotide sequences were recognized although lengths of analyzed nucleotide sequences were shorter: A(H3N2), 969 bases; B, 519 bases.

Clusters of influenza A virus (H1N1)pdm09 displaying 100% homology in hemagglutinin

Influenza A(H1N1)pdm09 comprised 38 clusters and displayed 100% homology. Of these, nine clusters of A(H1N1)pdm09 kept 100% homology for more than 30 days. One of these strains of A(H1N1)pdm09 maintained 100% homology for at least 94 days. Substituted amino acids in hemagglutinin were highlighted at 39 positions (Table 2).

Some Japanese and foreign strains (e.g. isolates in Mongolia and Thailand) in the international database could be allocated to the clusters of identical strains (Table 2).

DISCUSSION

A number of clusters of identical influenza A virus (H1N1)pdm09 strains were detected and the 100% homology lasted longer than a single epidemic season in Japan (3 months on average). It was not unusual that isolates in other areas of Japan and abroad were identical to those originating from the sentinel area of the present study.

Similar findings were reported also in France and New York State.[6, 13] In the United States multiple clades of influenza viruses entered and circulated with no clear pattern of spatial spread even in minor cities and towns with small populations (<100,000) as well as large cities.[7] The population size of the present study area was 425,000 as of September 1, 2014. Therefore, our results reflect the overall trend in Japan to a considerable extent and demonstrated that the clustering of identical strains is not the trend restricted to the sentinel area of this study.

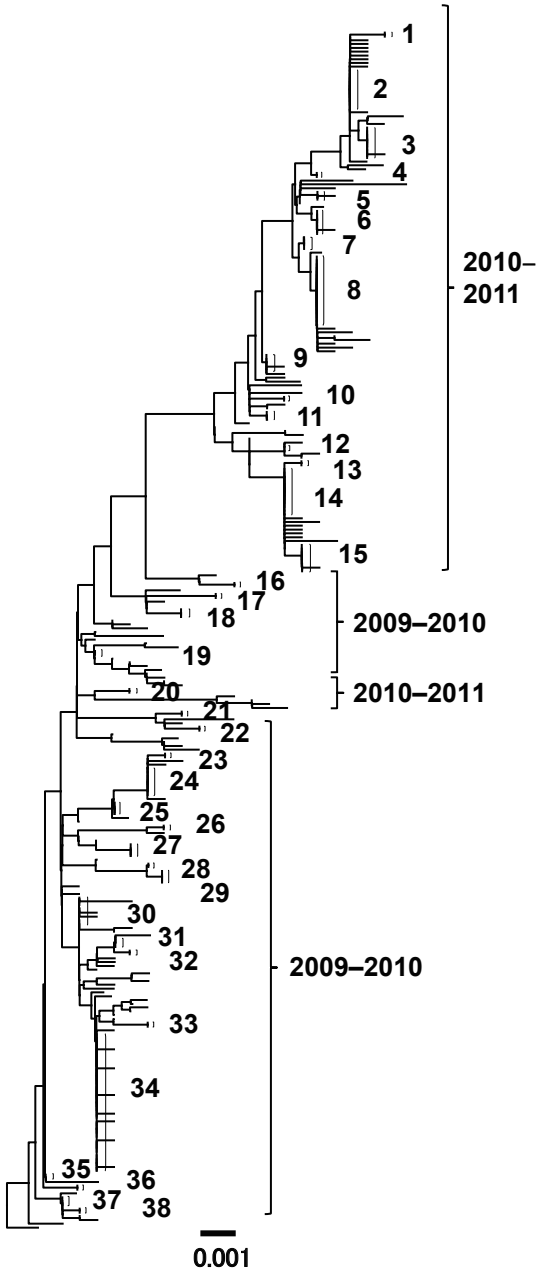

Fig. 1. Phylogenetic analysis of hemagglutinin of A(H1N1)pdm09. Half brackets with serial numbers indicate clusters of strains with 100% homology. An A(H1N1)pdm09 tree was composed of 38 of these clusters: no. 1–16 (isolated in the 2010–2011 season), no. 17–38 (2009–2010). A/South Carolina/1/1918(H1N1) was used as out-groups.

Table 2. Clusters of A(H1N1)pdm09 strains with identical amino acid sequences of hemagglutinin encompassing the position of A/California/07/2009, 1–567

Cluster*	Season	N†	Longest interval‡	Amino acid position from the first methionine§													
		Total		36	52	55	60	86	100	114	138	144	145	154	160	174	202
	A/California/07/2009			V	D	N	K	S	P	D	S	D	S	P	S	S	S
1	2010–2011	2	6	–	–	–	–	–	S	–	–	–	–	–	–	–	T
2	–	11	94	–	–	–	–	–	S	–	–	–	–	–	–	–	T
3	–	8	85	–	–	–	–	–	S	–	–	E	–	–	–	–	T
4	–	2	38	–	–	–	–	–	S	–	–	–	–	–	–	–	T
5	–	2	14	–	–	–	–	–	S	–	–	–	–	–	–	–	T
6	–	6	33	–	–	–	–	–	S	–	–	–	–	–	–	–	T
7	–	4	4	–	–	–	–	–	S	–	–	–	–	–	–	–	I
8	–	19	33	–	–	–	–	–	S	–	–	–	–	–	–	–	T
9	–	4	22	–	–	–	–	–	S	–	–	–	–	–	–	–	T
10	–	2	14	–	–	–	–	–	S	–	–	–	–	–	–	–	T
11	–	3	6	–	–	–	–	–	S	–	–	–	–	–	–	–	T
12	–	2‖	0	–	–	–	–	–	S	–	–	–	–	–	G	–	T
13	–	2	12	–	–	–	–	–	S	–	–	–	–	–	G	–	T
14	–	13	49	–	–	–	–	–	S	–	–	–	–	–	G	–	T
15	–	7	8	–	–	–	–	–	S	–	–	–	–	–	G	–	T
16	–	2	4	–	–	–	–	–	S	N	–	–	–	–	–	–	T
17	2009–2010	2	0	–	N	D	–	–	S	–	–	–	–	–	–	–	–
18	–	3	1	–	–	–	–	–	S	–	–	–	–	–	–	–	–
19	–	2¶	59	–	–	–	–	–	S	–	–	–	–	–	–	–	–
20	–	2**	20	–	–	–	–	–	S	–	–	–	–	–	–	–	–
21	–	2	3	–	–	–	–	–	S	–	–	–	L	–	–	–	–
22	–	2	5	–	–	–	–	–	S	–	–	–	–	–	–	–	–
23	–	2	1	–	N	–	–	L	S	–	–	–	–	–	–	–	–
24	–	8††	90	–	–	–	–	L	S	–	–	–	–	–	–	–	–
25	–	4	6	–	–	–	–	–	S	–	–	–	–	–	–	–	–
26	–	2	4	–	–	–	R	–	S	–	–	–	–	L	–	T	–
27	–	4	14	–	–	–	–	–	S	–	–	–	–	–	–	–	–
28	–	2	8	–	–	–	–	–	S	–	I	–	–	–	–	–	–
29	–	4	4	–	–	–	–	–	S	–	I	–	–	–	–	–	–
30	–	5	5	I	–	–	–	–	S	–	–	–	–	–	–	–	–
31	–	3	13	I	–	–	–	–	S	–	–	–	–	–	–	–	–
32	–	2	0	I	–	–	–	–	S	–	–	–	–	–	–	–	–
33	–	2	4	I	–	–	–	–	S	–	–	–	–	–	–	–	–
34	–	30	56	I	–	–	–	–	S	–	–	–	–	–	–	–	–
35	–	2‡‡	NS	–	–	–	–	–	S	–	–	–	–	–	–	–	–
36	–	2	6	–	–	–	–	–	S	–	–	–	–	–	–	–	–
37	–	3	4	–	–	–	–	–	S	–	–	–	–	–	–	–	–
38	–	2	4	–	–	–	–	–	S	–	–	–	–	–	–	–	–

*The serial number of a cluster composed of strains with identical amino acid sequences. †The number of strains in a cluster. ‡The longest interval (days) during which a pair of strains displayed 100% homology. §Positions containing substituted amino acids were highlighted in this Table. ‖A/Ulaanbaartar (Mongolia) /190/2011 is included in this cluster as one of the identical strains. ¶A/Hokkaido (Japan)/256/2009 included. **A/Thailand/0404–00–N0/2009 included. ††A/Tochigi (Japan)/350/2009 included. ‡‡A/Kobe (Japan)/91738/2009 included. NS: Duration could not be specified due to the unknown sampling date of the reference strain.

Continued on the following page

Table 2–Continued

Amino acid position from the first methionine§																									
207	214	220	239	245	262	291	302	311	314	321	338	343	379	391	468	472	496	499	516	524	526	537	544	545
S	A	S	D	N	T	D	A	N	P	P	I	S	A	E	S	N	V	G	E	S	R	V	V	V
–	T	T	–	D	–	–	–	D	–	–	V	–	–	K	N	–	–	–	K	–	–	–	–	–
–	T	T	–	–	–	–	–	D	–	–	V	–	–	K	N	–	–	–	K	–	–	–	–	–
–	T	T	–	–	–	–	–	D	–	–	V	–	–	K	N	–	–	–	K	–	–	–	–	–
–	T	T	–	–	–	–	–	D	–	–	V	–	–	K	N	–	–	–	K	–	–	–	–	–
–	T	T	–	–	–	–	–	D	–	–	V	–	–	K	N	–	–	–	K	–	–	–	–	–
–	T	T	–	–	N	–	–	D	–	–	V	–	–	K	N	–	–	–	K	–	–	–	–	–
–	T	T	–	–	–	–	–	D	–	–	V	–	–	K	N	–	–	–	K	–	–	–	–	–
–	T	T	–	–	–	–	–	D	–	–	V	–	–	K	N	–	–	–	K	–	–	–	–	–
–	T	T	–	–	–	–	–	–	–	–	V	–	–	K	N	–	–	–	–	–	–	–	–	–
–	T	T	–	–	–	–	S	–	–	–	V	–	–	K	N	–	–	–	–	–	–	–	I	–
–	T	T	–	–	–	–	–	–	–	–	V	F	–	K	N	–	–	–	–	–	–	–	–	–
–	T	T	–	–	–	–	–	–	–	–	V	–	–	K	N	–	–	E	–	–	–	–	–	–
–	T	T	–	–	–	–	–	–	–	–	V	–	–	K	N	–	–	–	–	–	–	–	–	–
–	T	T	–	–	–	–	–	–	–	–	V	–	–	K	N	–	–	–	–	–	–	–	–	–
–	T	T	–	–	–	–	–	–	–	–	V	–	–	K	N	–	–	–	–	P	–	–	–	–
–	–	T	–	–	–	–	–	–	–	–	V	–	–	K	N	–	–	–	–	–	–	–	–	I
–	–	T	–	–	–	–	–	–	–	–	V	–	–	K	–	–	–	–	–	–	–	–	–	–
–	–	T	–	–	–	–	–	–	–	–	V	–	T	K	–	–	–	–	–	–	G	–	–	–
–	T	T	–	–	–	–	–	–	–	–	V	–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	T	–	–	–	–	–	–	–	–	V	–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	T	E	–	–	–	–	–	S	–	V	–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	T	E	–	–	–	–	–	S	–	V	–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	T	–	–	–	N	–	–	–	–	V	–	–	K	–	–	–	–	–	–	–	–	–	–
–	–	T	–	–	–	N	–	–	–	–	V	–	–	K	–	–	–	–	–	–	–	–	–	–
–	–	T	–	–	–	N	–	–	–	–	V	–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	T	–	–	–	–	–	–	–	–	V	–	–	V	–	T	–	–	–	–	–	–	–	–
–	–	T	–	–	–	–	–	–	–	–	V	–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	T	–	–	–	–	–	–	–	S	V	–	–	–	–	–	I	–	–	–	–	–	–	–
–	–	T	–	–	–	–	–	–	–	S	V	–	–	V	–	–	I	–	–	–	–	–	–	–
–	–	T	–	–	–	–	–	–	–	–	V	–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	T	–	–	–	–	–	–	–	–	V	–	–	–	–	–	–	–	–	–	–	A	–	–
–	–	T	–	–	–	–	–	–	–	–	V	–	–	V	–	–	–	–	–	–	–	A	–	–
–	–	T	–	–	–	–	–	–	–	–	V	–	–	V	–	–	–	–	K	–	–	–	–	–
–	–	T	–	–	–	–	–	–	–	–	V	–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	T	–	–	–	–	–	–	–	–	V	–	–	–	–	–	–	–	–	–	–	–	–	–
N	–	T	–	–	–	–	–	–	–	–	V	–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	T	–	–	–	–	–	–	–	–	V	–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	T	–	–	–	–	–	–	–	–	V	–	–	–	–	–	–	–	–	–	–	–	–	–

Footnotes, refor to the previous page

As described above, a number of clusters were composed of identical A(H1N1)pdm09 strains and some of these were detected after a 30-day interval or longer in the present study. This result suggests that antigenic drift occurred infrequently in Japan during a single epidemic season, supporting the opinion that antigenic drift does not occur within the time frame of a single epidemic season in a single locality[5] as well as the hypothesis that genetic diversity is imported into temperate populations each epidemic season from the source population in which selection-driven antigenic drift is more efficient.[14] Tropical regions are one of the candidates for such a persistent influenza virus reservoir.[14, 15] A previous study assumed that East and Southeast Asia is such a reservoir that allows circulation via a region-wide network of temporally overlapping epidemics, and its seed strains reach Oceania, North America, Europe, and South America.[16]

Apart from the origins, antigenic drift has allowed viruses to escape from antibody-mediated neutralization through the accumulation of mutations in hemagglutinin segments. This escape was previously attributed to only seven positions in A(H3N2) hemagglutinin immediately adjacent to the receptor-binding site and such mutational sites were similarly detected among strains of influenza A(H1N1)pdm09.[17] Other studies identified several mutation sites in escape mutants from a monoclonal antibody *in vitro*,[18, 19] by the study of transmission between naïve ferrets,[20] and by a surveillance study[21]: K122N, N128D/S, D130E, K133T, G134S, K145N, K156E, K157N, G158E, N159/D/E/K, S186P, D190E/N, S210N, G228E, and K286M (H3 numbering: 14 subtracted from each number in Table 2). However, in the present study, we did not detect such amino acid substitutions in the positions observed previously during natural and experimental antigenic drift. This result suggests that such escape mutants were not generated in the sentinel area of the present study.

As described above, a number of clusters composed of identical strains of influenza A virus (H1N1)pdm09 have been isolated in five consecutive epidemic seasons, and some of these identical strains were detected after long intervals in Japan. This implies that influenza viruses mutate rarely in an epidemic season in Japan if it can be hypothesized, mutation frequency of influenza viruses are mostly the same among strains. These suggest that antigenic drift occurs infrequently in Japan, and genetic diversity may be introduced into the country from abroad each epidemic season. Results of the present study support the proposed 'sink-source' model of viral ecology[14] in which new lineages are seeded from a persistent influenza reservoir located in the tropics to sink populations in temperate regions including Japan.

Acknowledgments: The authors are grateful to the patients who agreed to participate in this study, and also Shimane Prefectural Institute of Public Health and Environment Science for providing MDCK cells. This study was supported by a Grant-in-Aid for Environmental Health Research from Tottori Prefecture, and a Grant-in-Aid for Education and Scientific Research from Tottori University.

The authors declare no conflict of interest.

REFERENCES

1 Nair H, Brooks WA, Katz M, Roca A, Berkley JA, Madhi SA, et al. Global burden of respiratory infections due to seasonal influenza in young children: a systematic review and meta-analysis. Lancet. 2011;378:1917-30. PMID: 22078723.
2 Lang PO, Govind S, Mitchell WA, Siegrist CA, Aspinall R. Vaccine effectiveness in older individuals: what has been learned from the influenza-vaccine experience. Ageing Res Rev. 2011;10:389-95. PMID: 20888439.
3 Monto AS, Ansaldi F, Aspinall R, McElhaney JE, Montano LF, Nichol KL, et al. Influenza control in the 21st century: Optimizing protection of older adults. Vaccine. 2009;27:5043-53. PMID: 19559118.
4 Hak E, F. W, Nordin J, Mullooly J, Poblete S, Nichol KL. Development and validation of a clinical prediction rule for hospitalization due to pneumonia or influenza or death during influenza epidemics among community-dwelling elderly persons. J Infect Dis. 2004;189:450-8. PMID: 14745702.
5 Nelson MI, Holmes EC. The evolution of epidemic influenza. Nat Rev Genet. 2007;8:196-205. PMID: 17262054.
6 Lavenu A, Leruez-Ville M, Chaix ML, Boelle PY, Rogez S, Freymuth F, et al. Detailed analysis of the genetic evolution of influenza virus during the course of an epidemic. Epidemiol Infect. 2006;134:514-20. PMID: 16316493.
7 Nelson MI, Edelman L, Spiro DJ, Boyne AR, Bera J, Halpin R, et al. Molecular epidemiology of A/H3N2 and A/H1N1 influenza virus during a single epidemic season in the United States. PLoS Pathog. 2008;4:e1000133. PMID: 18725925.
8 Webster R, Cox N, Stohr K. WHO Manual on Animal Influenza Diagnosis and Surveillance. World Health Organization DoCDSaR, editor. Geneva: World Health Organization; 2002. p. 1-97.
9 Zhou B, Donnelly ME, Scholes DT, St George K, Hatta M, Kawaoka Y, et al. Single-reaction genomic amplification accelerates sequencing and vaccine production for classical and Swine origin human influenza a viruses. J Virol. 2009;83:10309-13. PMID: 19605485.
10 Mahony JB, Chong S, Luinstra K, Petrich A, Smieja M. Development of a novel bead-based multiplex PCR assay for combined subtyping and oseltamivir resistance genotyping (H275Y) of seasonal and pandemic H1N1 influenza A viruses. J Clin Virol. 2010;49:277-82. PMID: 20846903.
11 Dawood FS, Jain S, Finelli L, Shaw MW, Lindstrom S, Garten RJ, et al. Emergence of a novel swine-origin influenza A (H1N1) virus in humans. N Engl J Med. 2009;360:2605-15. PMID: 19423869.
12 Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol Biol Evol. 2013;30:2725-9. PMID: 24132122.
13 Nelson MI, Simonsen L, Viboud C, Miller MA, Taylor J, George KS, et al. Stochastic processes are key determinants

of short-term evolution in influenza a virus. PLoS Pathog. 2006;2:e125. PMID: 17140286.

14 Rambaut A, Pybus OG, Nelson MI, Viboud C, Taubenberger JK, Holmes EC. The genomic and epidemiological dynamics of human influenza A virus. Nature. 2008;453:615-9. PMID: 18418375.

15 Viboud C, Alonso WJ, Simonsen L. Influenza in tropical regions. PLoS Med. 2006;3:e89. PMID: 16509764.

16 Russell CA, Jones TC, Barr IG, Cox NJ, Garten RJ, Gregory V, et al. The global circulation of seasonal influenza A (H3N2) viruses. Science. 2008;320:340-6. PMID: 18420927.

17 Koel BF, Burke DF, Bestebroer TM, van der Vliet S, Zondag GC, Vervaet G, et al. Substitutions near the receptor binding site determine major antigenic change during influenza virus evolution. Science. 2013;342:976-9. PMID: 24264991.

18 Rudneva I, Ignatieva A, Timofeeva T, Shilov A, Kushch A, Masalova O, et al. Escape mutants of pandemic influenza A/H1N1 2009 virus: variations in antigenic specificity and receptor affinity of the hemagglutinin. Virus Res. 2012;166:61-7. PMID: 22459010.

19 O'Donnell CD, Vogel L, Wright A, Das SR, Wrammert J, Li GM, et al. Antibody pressure by a human monoclonal antibody targeting the 2009 pandemic H1N1 virus hemagglutinin drives the emergence of a virus with increased virulence in mice. MBio. 2012;3:1-10. PMID: 22647789.

20 Guarnaccia T, Carolan LA, Maurer-Stroh S, Lee RT, Job E, Reading PC, et al. Antigenic drift of the pandemic 2009 A(H1N1) influenza virus in A ferret model. PLoS Pathog. 2013;9:e1003354. PMID: 23671418.

21 Strengell M, Ikonen N, Ziegler T, Julkunen I. Minor changes in the hemagglutinin of influenza A(H1N1)2009 virus alter its antigenic properties. PLoS One. 2011;6:e25848. PMID: 22022458.