Virus Genes (2013) 46:323–329
DOI 10.1007/s11262-012-0868-9

Surveillance and characterization of avian influenza viruses from migratory water birds in eastern Hokkaido, the northern part of Japan, 2009–2010

Lary N. B. Abao · Dulamjav Jamsransuren · Vuong N. Bui · Lai H. Ngo · Dai Q. Trinh · Emi Yamaguchi · Dhanasekaran Vijaykrishna · Jonathan Runstadler · Haruko Ogawa · Kunitoshi Imai

Received: 13 September 2012 / Accepted: 11 December 2012 / Published online: 21 December 2012

Abstract Avian influenza virus (AIV) surveillance was conducted around a small pond in Obihiro, eastern Hokkaido, Japan. Eleven AIVs were isolated from a total of 1,269 fecal samples of migratory wild birds collected during 2009 and 2010. The sample number covered approximately 60 % of the total number of birds observed during sampling periods. The subtypes of the isolates included H3N8 (4 isolates), H5N2 (3), H6N2 (2), H6N1 (1), and H11N2 (1). The H3N8 subtype was most prevalent as in the previous studies performed in Hokkaido. The three H5N2 isolates genetically characterized as low pathogenic AIV were closely related to the strains previously isolated from aquatic wild birds in Japan and also to the Korean strains isolated from aquatic birds in recent years. In Korea, H5N2 subtype virus has often been isolated from poultry and wild birds, as well as reassortant viruses generated from duck H5N2 viruses and chicken H9N2 virus, and avian-swine-like reassortant H5N2 viruses. Considering the previous chicken outbreaks caused by highly pathogenic H5N2 viruses, which affected many countries, it should be an important priority to continue, monitoring the evolution of H5N2 viruses circulating in the region.

Keywords Avian influenza virus · Surveillance · H5N2 · H3N8 · H6N2 · H6N1 · H11N2

L. N. B. Abao · D. Jamsransuren · V. N. Bui · L. H. Ngo · D. Q. Trinh · E. Yamaguchi · H. Ogawa (✉) · K. Imai
Research Center for Animal Hygiene and Food Safety, Obihiro University of Agriculture and Veterinary Medicine, 2-11 Inada, Obihiro, Hokkaido 080-8555, Japan
e-mail: hogawa@obihiro.ac.jp

D. Vijaykrishna
Program of Emerging Infectious Diseases, Duke-NUS Graduate Medical School, Singapore 169857, Singapore

J. Runstadler
Department of Biological Engineering and Division of Comparative Medicine, Massachusetts Institute of Technology, Cambridge, MA 02139, USA

Introduction

It is recognized that wild waterfowl serve as a natural reservoir of avian influenza virus (AIV), and have the potential to transmit viruses along their migratory pathways [1]. Recent studies have confirmed that wild birds play important roles in the global movement of AIVs including the highly pathogenic avian influenza (HPAI) H5N1 viruses [2–4]. Japan lies along the migration route of wild birds, particularly in the East Asia–Australia Shorebird Flyway [3, 4], and is vulnerable to the introduction of HPAI and low pathogenic avian influenza (LPAI) strains via migratory species. In fact, Japan has experienced HPAI H5N1 outbreaks repeatedly in recent years including the worst outbreak experienced during the winter season between 2010 and 2011 [5, 6]. In the most recent case, H5N1 viruses were also isolated from several wild birds [5], implicating the role of migratory water birds in these outbreaks. Under the threat of such outbreaks, continued AIV surveillance in wild birds should be regarded as one of the important control measures for fighting against avian influenza.

HPAI virus (HPAIV) of the H5N1 subtype has had tremendous effects on wide regions of the world since the virus first appeared in 1997 [7]. High mortality caused by the HPAI H5N1 virus in poultry and hundreds of human deaths raised concern for the appearance of a new

pandemic influenza virus. The H5N1 virus may gain pandemic potential by acquiring adaptation to humans through mutation and/or genetic reassortment, as described recently [8, 9]. Extensive efforts have been devoted to influenza surveillance worldwide to understand the ecological and evolutionary processes that govern the development of influenza viruses with pandemic potential–including the geographic distribution of H5N1 viruses and the genetic changes in those viruses [10, 11]. Those research studies have provided substantial amounts of new information on influenza ecology that are being used in effective disease control [10, 12, 13], but continued efforts to understand more about the evolution of influenza virus are necessary.

In Japan, AIV surveillance studies have mainly been performed in three districts, Hokkaido [5, 14], Tohoku [15, 16], and San-in [17–19]. In recent years, we have been performing AIV surveillance studies in eastern Hokkaido where such studies have been rare. In our previous work [20, 21], samples of wild birds were collected around seashores, lakes near the sea, and rivers where hundreds and thousands of birds congregate. From those samples, we isolated AIVs that included viral subtypes rarely isolated in other parts of Japan. The isolates also included reassortant viruses that could cause severe respiratory disease in mice [20, 21]. In the current study, surveillance was conducted at a pond located inland in Hokkaido, where certain species of migratory wild birds can be found during fall. In this study, several subtypes of AIVs, including H5N2, were isolated and genetically analyzed.

Materials and methods

Sample collection

A total of 1,269 fecal samples from migratory water birds, mainly ducks, in the Hakuryo Pond of Obihiro University of Agriculture and Veterinary Medicine (GPS coordinates 42°52′N, 143°10′E) located in Obihiro City were collected during the middle of September to the beginning of December during 2009 and 2010. The pond is 1,300 square meters in area, and an average of 130 birds was found each day during the sampling period. Sample collection was conducted over several days interval (average of 5 days) at each time point. Only samples that appeared fresh were collected for the purpose of virus isolation, and the number of samples collected was kept proportionally below the number of birds found in the pond on each day of collection (average of 60 %). The fecal samples were suspended in virus transport medium (VTM) to prepare 20 % homogenates. The VTM was comprised of minimum essential medium (MEM: pH 7.2–7.4, Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) that was supplemented with 0.5 % bovine serum albumin and antimicrobials as we previously described [20]. The homogenates were kept at room temperature for <2 h, and then stored at −80 °C until use.

Virus isolation

The fecal homogenates in VTM were thawed and centrifuged at 12,000×*g* for 3 min at 4 °C. Then, 0.1 ml of each supernatant was inoculated into the allantoic cavity of 9 to 11-day-old embryonating chicken eggs (two eggs per homogenate sample). The eggs were incubated at 37 °C for 4 days, and then chilled at 4 °C. The allantoic fluids (AFs) collected from the eggs were tested by a hemagglutination test with 0.5 % chicken red blood cells. The AF samples that did not show hemagglutination activity were passaged to a second egg inoculation followed by a hemagglutination test.

Real-time reverse transcription-polymerase chain reaction (RRT-PCR)

Total RNA was extracted from the AFs with hemagglutination activity using ISOGEN-LS (Nippon Gene, Tokyo, Japan) according to the manufacturer's instruction. First-strand cDNA was produced using random hexamer primers (Invitrogen, Carlsbad, CA) and M-MLV reverse transcriptase (Invitrogen) under the following conditions: 25 °C for 10 min, 37 °C for 50 min, and 65 °C for 10 min. RRT-PCR for the M gene of the influenza A virus was carried out using Taqman Universal PCR Master mix (Applied Biosciences, Foster City, CA) in the ABI PRISM Sequence Detection System 7900HT (Applied Biosciences) as we previously described [20].

AIV subtyping

The AF samples determined to be positive with the M gene were subtyped by RT-PCR using 15 sets of H (H1–H15) primers and 9 sets of N (N1–N9) primers according to the previous reports by Lee et al. [22], and Qiu et al. [23], respectively. The PCR was performed using Takara Ex Taq (Takara Bio Inc., Shiga, Japan) under the following conditions: 5 min at 95 °C; 40 cycles of denaturation for 30 s at 95 °C, 30 s of annealing at 50–60 °C, and 30 s of extension at 72 °C; and one cycle of extension at 72 °C for 10 min. The annealing temperatures for the primer sets included: 50 °C for H1, H5, H6, H7, H8, H9, H10, H12, and H13; 55 °C for other H primers; and 60 °C for all the N-primer sets. PCR products obtained were electrophoresed on a 1–2 % agarose gel stained with ethidium bromide and visualized under UV light.

Nucleotide sequencing

For nucleotide sequencing of the AIV isolates, the viral RNA was transcribed into cDNA using the Uni12 primer (5′-agcraaagcagg-3′) and Superscript III Reverse Transcriptase (Invitrogen) at 42 °C for 60 min followed by 70 °C for 10 min. Using the cDNAs as templates, PCR was conducted to amplify a full-length of the M, HA, and NA genes by the method reported by Hoffmann et al. [24]. The PCR products obtained were separated by 1 % agarose gel electrophoresis and purified using a QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). The purified PCR products were sequenced using a BigDye terminator ver. 3.1 Cycle Sequencing Kit (Applied Biosystems) and analyzed with the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). The primer sets described above and additional walking primers designed by us (sequences available upon request) were used to obtain the full-length nucleotide sequence of the M, HA, and NA genes.

Phylogenetic analysis

The nucleotide sequences obtained were analyzed with the Genetyx Ver. 9 software (GENETYX Corp., Tokyo, Japan) and compared with other sequences available in GenBank by BLAST homology searches (http://www.ncbi.nlm.nih.gov/genomes/ FLU/FLU.html). The sequences were aligned manually using Se-Al (http://tree.bio.ed.ac.uk/software/seal/) and the best-fit evolutionary model for each dataset was estimated using JModeltest [25]. Phylogenetic trees for each gene datasets were generated using the best-fit evolutionary model and the maximum likelihood method in PhyML 3.0 software [26]. Phylogenetic supports were also estimated using the maximum likelihood bootstrap method in PhyML 3.0.

Results

Virus isolation and AIV subtyping

Among the 1,269 fecal samples of migratory water birds in the Hakuryo Pond in Obihiro City, 11 samples (0.87 %) collected between September and December in 2009 and 2010 were determined to be positive with the M gene of the influenza A virus in the RRT-PCR. The threshold cycle (Ct) values in the RRT-PCR and the hemagglutination titer for the AFs of the eggs inoculated with the 11 samples ranged from 15.98–25.77 and 1:64–1:1024, respectively. Subtypes of the isolated AIVs included H3N8 (4 isolates), H5N2 (3), H6N1 (1), H6N2 (2), and H11N2 (1) (Table 1).

Characterization of the M gene of the AIV isolates

The full-length nucleotide sequences of the M gene were analyzed for the 11 AIV isolates. The nucleotide sequences obtained in the current study are available in GenBank under the accession numbers given in Table 1. Phylogenetic analyses of the obtained sequences revealed that all M segments belong to the Eurasian lineage, which can be grouped into sublineages 1 and 2. In the Eurasian sublineage 2, all 3 isolates of H5N2 viruses, 1 of the 2 isolates of

Table 1 AIV isolates obtained in surveillance using fecal samples of migratory water birds in Obihiro during 2009–2010

Virus strains	Subtype	Date of sample collection[a]	Ct value[b]	HA titer[c]	Accession number (M, NA, HA)[d]
A/avian/Japan/9UO004/2009	H6N1	10/15/09	16.90	1:1024	JX673927
A/avian/Japan/9UO025/2009	H5N2	10/15/09	16.96	1:256	JX673935, JX673936, JX673937
A/avian/Japan/9UO036/2009	H5N2	10/15/09	17.21	1:128	JX673922, JX673923, JX673924
A/avian/Japan/9UO139/2009	H5N2	11/04/09	18.08	1:256	JX673928, JX673929, JX673930
A/avian/Japan/9UO203/2009	H11N2	11/13/09	19.70	1:128	JX673932
A/avian/Japan/9UO238/2009	H6N2	11/13/09	16.88	1:1024	JX673934
A/avian/Japan/9UO260/2009	H6N2	11/18/09	15.98	1:256	JX673921
A/avian/Japan/10UO053/2010	H3N8	09/24/10	21.90	1:256	JX673933
A/avian/Japan/10UO057/2010	H3N8	09/24/10	18.60	1:128	JX673931
A/avian/Japan/10UO143/2010	H3N8	10/01/10	25.77	1:128	JX673925
A/avian/Japan/10UO178/2010	H3N8	10/01/10	17.24	1:64	JX673926

[a] Day/month/year

[b] Threshold cycle (Ct) values obtained for the AF samples in the RRT-PCR for the M gene of influenza A virus

[c] Hemagglutination titer for the AF samples

[d] GenBank accession numbers of the full-length M gene for all the isolates. For the H5N2 isolates, the accession numbers of the full-length HA and NA genes are also listed

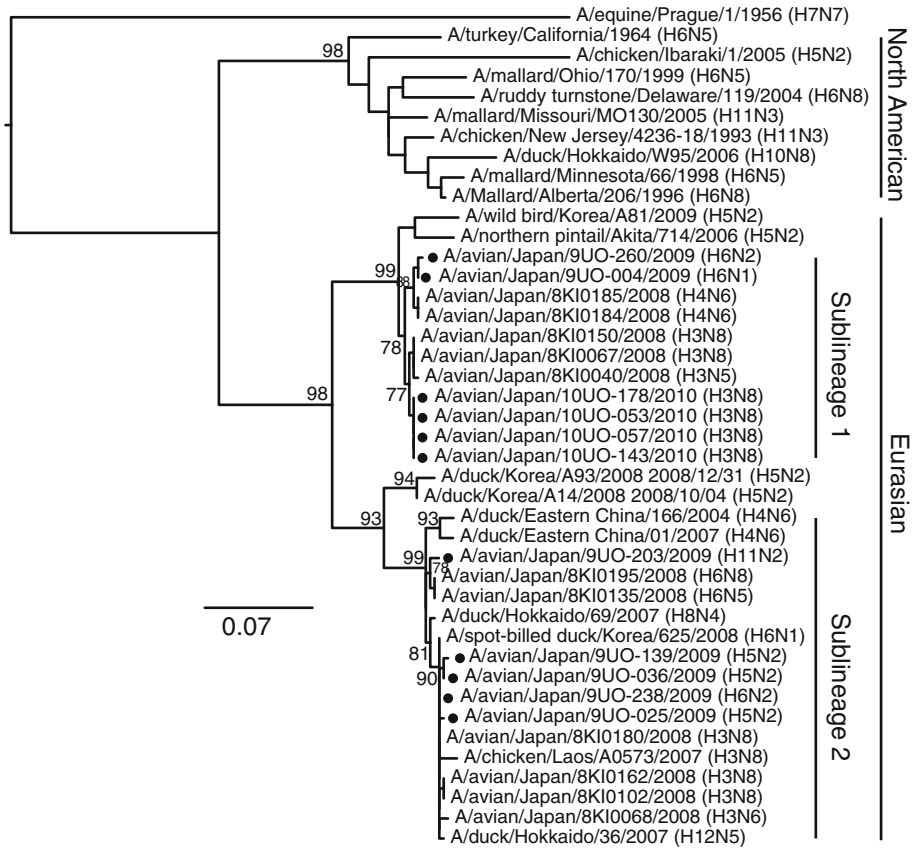

Fig. 1 Phylogenetic tree of the M gene of AIVs. Full-length nucleotide sequences of the M genes of the H5N2 isolated in this study were phylogenetically analyzed in comparison with the sequences available in GenBank. The H5N2 strains isolated in this study are marked with *closed circles*. A/Equine/Prague/1/1956 was used to root the tree. *Numbers* above/below branches represent bootstrap values (1,000 replicates). Only highly supported branches with >70 % bootstrap values are shown. *Scale bars* represent nucleotide substitutions per site

H6N2 viruses, and 1 isolate of the H11N2 virus were included. In the Eurasian sublineage 1, all the 4 isolates of the H3N8 viruses and 1 isolate each of the H6N1 and H6N2 viruses were included (Fig. 1).

Genetic characterization of the H5N2 AIV isolates

To further characterize the 3 isolates of H5N2 viruses, full-length sequences of the HA and NA genes were analyzed. The 3 H5N2 isolates shared identities of 99.5–100.0 and 99.1–100.0 % in nucleotide and amino acid sequence, respectively (data not shown). All of the H5N2 viruses shared a common motif (PQKETR/GLF) at the cleavage site of the HA protein. The phylogenetic tree for the H5 genes also indicated that the 3 isolates of the H5N2 viruses were genetically similar to each other. The HA genes of the H5N2 isolates were closely related to the A/duck/Tsukuba/189/2008 (H5N2) isolated in Japan, and also to the H5N2 viruses isolated from aquatic birds in Korea in 2007 (Fig. 2). In the tree, the H5N2 viruses isolated in the current study were found in the same cluster with most of the H5N2 viruses previously isolated from wild birds in Japan. However, A/chicken/Ibaraki/1/2005 and related strains, which were isolated from chickens in Ibaraki prefecture in 2005 and 2006, were allocated to a separate cluster. In addition, A/duck/Tsukuba/168/2005, also isolated in Japan, formed a small cluster with the Korean strains isolated from a duck in 2004 and from a wild bird in 2008 and falling between the two other clusters (Fig. 2). Phylogenetic analysis for the NA genes showed similar results (Fig. 3).

Nucleotide homologies in the HA and NA genes were analyzed between A/avian/Japan/9UO036/2009 and other H5N2 strains previously isolated in Japan. The HA and NA genes of A/avian/Japan/9UO036/2009 shared over 97 % nucleotide identity with those of the H5N2 strains isolated from wild birds in Akita, Niigata, Shimane, and Tsukuba between 2006 and 2008. However, the HA and NA genes of A/avian/Japan/9UO036/2009 shared 82.3 and 83.6 % identity with those of A/duck/Tsukuba/168/2005, respectively. Nucleotide identity in the HA and NA genes was approximately 79 and 81 % between A/avian/Japan/9UO036/2009 and A/chicken/Ibaraki/1/2005 or A/chicken/Ibaraki/17/2006, respectively.

Discussion

In the current study, we conducted the first AIV surveillance within Obihiro City, in eastern Hokkaido, Japan. The fecal samples of migratory water birds were collected around a small pond in the city during September to December.

Fig. 2 Phylogenetic tree of the HA genes of AIVs. Full-length nucleotide sequences of the HA genes of the H5N2 isolated in this study were phylogenetically analyzed in comparison with the sequences available for the H5 subtype virus except H5N1 viruses in GenBank. The H5N2 strains isolated in this study are marked with *closed circles*, A/duck/Tsukuba/168/2005 with a *closed square*, and other Japanese strains with *open triangles*. *Numbers* above/below branches represent bootstrap values (1,000 replicates). Only highly supported branches with >70 % bootstrap values are shown. *Scale bars* represent nucleotide substitutions per site

Among the 1,269 fecal samples collected over two years, 11 strains of AIV were obtained with an isolation rate of 0.87 %. The M genes of the 11 isolates belonged to the Eurasian lineage, which can be grouped into sublineages 1 and 2 (Fig. 1). In our previous study, 18 strains of AIV were isolated from 1,039 fecal samples (1.7 %). In that study, the samples were collected along a riverside in eastern Hokkaido near Obihiro City between October and February, but all the 18 isolates were obtained from the 720 samples collected between October and December (2.5 % prevalence) [20]. Other groups have been performing surveillance studies on migratory birds mainly in three districts of Japan, where prevalence between 0.43 and 5.86 % was reported [14–19] and the prevalence in the current study (0.87 %) and our

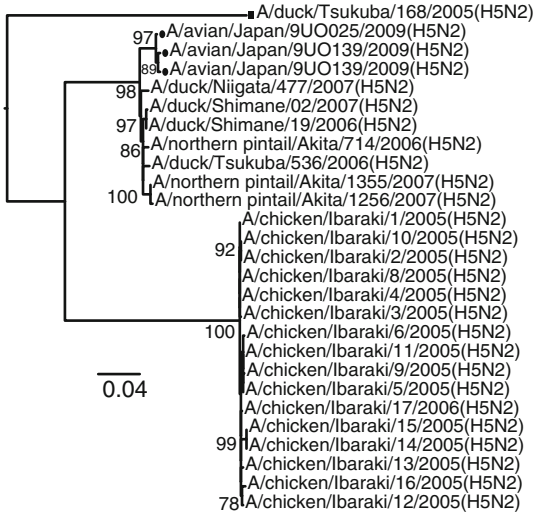

Fig. 3 Phylogenetic tree of the NA genes of AIVs. Full-length nucleotide sequences of the NA genes of the H5N2 isolated in this study were phylogenetically analyzed in comparison with the sequences available for the H5N2 viruses isolated in Japan. The three strains isolated in this study are marked with *closed circle*s, and A/duck/Tsukuba/168/2005 with a *closed square*. *Numbers* above/below branches represent bootstrap values (1,000 replicates). Only highly supported branches with >70 % bootstrap values are shown. *Scale bars* represent nucleotide substitutions per site

previous studies (1.7 and 2.5 %) [20] fall within this range. The highest rate in prior studies was reported on samples collected in central and northern Hokkaido during October and November [14]. In that study, a total of 3,718 samples were collected in eight consecutive years, and 218 AIV isolates were obtained from those. The number of isolates per year ranged from 7 to 63 in the 8 years, although the sample number in each year is not provided. In other studies in Japan, AIV isolation rates of 1.84 % (38 isolates/2,066 samples, 2006–2007) and 1.05 % (25/2,381, 2007–2008) in Tohoku district [15, 16], and 0.43 % (2/465, 1980–1981), 1.47 % (17/1,156, 1981–1982), and 1.28 % (18/1404, 1997–2000) in San-in district [17–19] were reported. In all the studies including ours, egg inoculation and AIV subtyping were conducted in a similar way [14–20]; therefore the variations in the prevalence might be attributed to the sampling location, year, or other factors. Recently, Hoye et al. [10] critically reviewed nearly 200 papers on AIV surveillance in wild birds with an aim to distill considerations pertinent to surveillance, and emphasized the importance of what, when, where, and how many to sample in the context of survey objectives. In order to achieve effective AIV surveillance especially for HPAIV monitoring, it should be important to take into account factors such as survey design, sampling, and interpretation in the context of host populations. Although the sample size was relatively small in the current study, the sample number covered approximately 60 % of the bird number found in the pond during the testing period. Therefore, the results obtained in this study are likely to reflect the whole population of the bird group who stopped over during migration at the pond.

Subtypes of the 11 AIVs in this study included H3N8, H5N2, H6N2, H6N1, and H11N2. Among those, the H3N8 subtype was most prevalent (Table 1), which was consistent with the results in the previous studies performed in Hokkaido [14, 20]. In another respect, the H11N2 virus recovered in the current study was the second isolate of this subtype in Japan for which sequence data has been submitted to GenBank. In addition, few H5N2 subtype viruses have been isolated from wild birds in Japan. The nucleotide sequences of 14 H5N2 viruses isolated from aquatic birds in Japan are currently available in the GenBank database. The H5N2 viruses with the highest nucleotide identity to the HA gene and NA gene of the H5N2 isolates obtained in the current study were A/duck/Tsukuba/189/2008, and A/wild bird/Korea/A81/2009, respectively. Phylogenetic analysis of the HA gene clearly indicated that the H5N2 isolates were genetically close to A/duck/Tsukuba/189/2008 and the H5N2 aquatic bird strains isolated in Korea in 2007. The cluster also included other H5N2 viruses previously isolated from wild birds in Japan. However, A/duck/Tsukuba/168/2005 and 16 chicken H5N2 strains isolated in Ibaraki were grouped with two separate lineages of viruses (Fig. 2). Phylogenetic analysis on the NA gene showed similar results (Fig. 3). The 16 Ibaraki strains were isolated during a chicken outbreak, in which 5.78 million chickens were culled [27]. The Ibaraki H5N2 viruses were found to be closely related to the H5N2 LPAI viruses prevalent in Central America [28]. It should be noted that HPAI H5N2 viruses have caused poultry outbreaks in the United States [28, 29], Mexico [30, 31], Italy [32], and South Africa [33]. Importantly, LPAI H5N2 viruses had been isolated before the HPAI H5N2 outbreak in most countries [29–31, 33, 34]. In the current study, we isolated H5N2 viruses in Obihiro City, which is surrounded by an area of animal husbandry including poultry farming. Considering the history of H5N2 viruses and our current knowledge of viral evolution, it should be an important priority to prevent contact between the poultry and wild birds that may carry the AIVs of H5 subtype regardless of their pathogenicity. This study points out the importance of surveillance in areas where humans, agriculture, and wild birds all interface.

It was also striking to find that the H5N2 strains isolated in the current study were closely related to the strains isolated in Korea, where the H5N2 subtype virus has often been isolated from poultry and wild birds in recent years [35, 36]. Three H9N2 viruses obtained at live bird markets and duck farms in Korea were found to be reassortant viruses generated from the H5N2 viruses of domestic ducks and the H9N2 virus endemic in Korean chickens. The

reassortant H9N2 virus was confirmed to replicate well in chickens without preadaptation, though the original H9N2 virus did not replicate well [36]. More importantly, the H5N2 viruses recently isolated from pigs in Korea included not only an entirely avian-like virus but also an avian-swine-like reassortant with several genes from a swine H3N1-like virus. The reassortant virus was more adapted and more readily transmitted to pigs than the purely avian-like virus [37]. Studies like the one reported here will be important to continue monitoring the evolution of H5N2 viruses circulating in Korea and surrounding countries, including Japan. This study may prove critical to controlling the spread of novel viruses that might cause detrimental outbreaks in poultry and/or a new pandemic in humans.

Acknowledgments We would like to thank Eric Bortz (Mount Sinai School of Medicine and currently the University of Alaska Anchorage) for his help on the sequence analysis and data management. We are grateful for the technical support of Sachiko Matsuda. This study was partially supported by grants from the Program of Founding Research Centers for Emerging and Re-emerging Infectious Diseases and by a Grant-in-Aid for Exploratory Research (19659115) from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan. This study was partially supported by the US National Institute of Allergy and Infectious Diseases (NIAID contracts HHSN266200700009C and HHSN266200700007C).

References

1. R.G. Webster, W.J. Bean, O.T. Gorman, T.M. Chambers, Y. Kawaoka, Microbiol. Rev. **56**, 152–179 (1992)
2. H. Chen, G.J.D. Smith, S.Y. Zhang, K. Qin, J. Wang, K.S. Li, R.G. Webster, J.S.M. Peiris, Y. Guan, Nature **436**, 191–192 (2005)
3. D. Normile, Science **310**, 426–428 (2005)
4. B. Olsen, V.J. Munster, A. Wallensten, J. Waldenstrom, A.D. Osterhaus, R.A. Fouchier, Science **312**, 384–388 (2006)
5. Y. Sakoda, H. Ito, Y. Uchida, M. Okamatsu, N. Yamamoto, K. Soda, N. Nomura, S. Kuribayashi, S. Shichinohe, Y. Sunden, T. Umemura, T. Usui, H. Ozaki, T. Yamaguchi, T. Murase, T. Ito, T. Saito, A. Takada, H. Kida, J. Gen. Virol. **93**, 541–550 (2012)
6. K. Sugiura, M. Yamamoto, T. Nishida, D. Tsukamoto, T. Saito, T. Onodera, Rev. Sci. Tech. **28**, 1005–1013 (2009)
7. P.K. Chan, Clin. Infect. Dis. **34**(Suppl 2), S58–S64 (2002)
8. M. Imai, T. Watanabe, M. Hatta, S.C. Das, M. Ozawa, K. Shinya, G. Zhong, A. Hanson, H. Katsura, S. Watanabe, C. Li, E. Kawakami, S. Yamada, M. Kiso, Y. Suzuki, E.A. Maher, G. Neumann, Y. Kawaoka, Nature **486**, 420–428 (2012)
9. S. Herfst, E.J. Schrauwen, M. Linster, S. Chutinimitkul, E. De Wit, V.J. Munster, E.M. Sorrell, T.M. Bestebroer, D.F. Burke, D.J. Smith, G.F. Rimmelzwaan, A.D. Osterhaus, R.A. Fouchier, Science **336**, 1534–1541 (2012)
10. B.J. Hoye, V.J. Munster, H. Nishiura, M. Klaassen, R.A. Fouchier, Emerg. Infect. Dis. **16**, 1827–1834 (2010)
11. W.G. Dundon, A. Heidari, A. Fusaro, I. Monne, M.S. Beato, G. Cattoli et al., Vet. Microbiol. **154**, 209–221 (2012)
12. A. Globig, A. Baumer, S. Revilla-Fernández, M. Beer, E. Wodak, M. Fink et al., Emerg. Infect. Dis. **15**, 1633–1636 (2009)
13. C.J. Feare, Avian Dis. **54**, 201–212 (2010)
14. R. Manzoor, Y. Sakoda, A. Mweene, Y. Tsuda, N. Kishida, G.R. Bai, K. Kameyama, N. Isoda, K. Soda, M. Naito, H. Kida, Virus Genes **37**, 144–152 (2008)
15. A. Jahangir, S. Ruenphet, S. Ueda, Y. Ueno, D. Shoham, J. Shindo, M. Okamura, M. Nakamura, K. Takehara, Virus Res. **143**, 44–52 (2009)
16. A. Jahangir, Y. Watanabe, O. Chinen, S. Yamazaki, K. Sakai, M. Okamura, M. Nakamura, K. Takehara, Avian Dis. **52**, 49–53 (2008)
17. Y. Fujimoto, H. Ito, S. Shivakoti, J. Nakamori, R. Tsunekuni, K. Otsuki, T. Ito, J. Vet. Med. Sci. **72**, 963–967 (2010)
18. K. Otsuki, O. Takemoto, R. Fujimoto, Y. Kawaoka, M. Tsubokura, Zentralbl. Bakteriol. Mikrobiol. Hyg. A. **265**, 235–242 (1987)
19. Y. Shengqing, K. Shinya, K. Otsuki, H. Ito, T. Ito, J. Vet. Med. Sci. **64**, 1049–1052 (2002)
20. V.N. Bui, H. Ogawa, K. Karibe, K. Matsuo, T.H. Nguyen, S.S. Awad, G.L. Minoungou, Xininigen, K. Saito, Y. Watanabe, J.A. Runstadler, G.M. Happ, K. Imai, J. Vet. Med. Sci. **73**, 209–215 (2011)
21. V.N. Bui, H. Ogawa, Xininigen, K. Karibe, K. Matsuo, S.S. Awad, G.L. Minoungou, S. Yoden, H. Haneda, L.H. Ngo, S. Tamaki, Y. Yamamoto, K. Nakamura, K. Saito, Y. Watanabe, J. Runstadler, F. Huettmann, G.M. Happ, K. Imai, Virology **423**, 77–88 (2012)
22. M.S. Lee, P.C. Chang, J.H. Shien, M.C. Cheng, H.K. Shieh, J. Virol. Methods **97**, 13–22 (2001)
23. B.F. Qiu, W.J. Liu, D.X. Peng, S.L. Hu, Y.H. Tang, X.F. Liu, J. Virol. Methods **155**, 193–198 (2009)
24. E. Hoffmann, J. Stech, Y. Guan, R.G. Webster, D.R. Perez, Arch. Virol. **146**, 2275–2289 (2001)
25. D. Posada, Mol. Biol. Evol. **25**, 1253–1256 (2008)
26. S. Guindon, J.F. Dufayard, V. Lefort, M. Anisimova, W. Hordijk, O. Gascuel, Syst. Biol. **59**, 307–321 (2010)
27. M. Okamatsu, T. Saito, Y. Yamamoto, M. Mase, S. Tsuduku, K. Nakamura, K. Tsukamoto, S. Yamaguchi, Vet. Microbiol. **124**, 35–46 (2007)
28. W.J. Bean, Y. Kawaoka, J.M. Wood, J.E. Pearson, R.G. Webster, J. Virol. **54**, 151–160 (1985)
29. Y. Kawaoka, C.W. Naeve, R.G. Webster, Virology **139**, 303–316 (1984)
30. M. Garcia, J.M. Crawford, J.W. Latimer, E. Rivera-Cruz, M.L. Perdue, J. Gen. Virol. **77**, 1493–1504 (1996)
31. T. Horimoto, E. Rivera, J. Pearson, D. Senne, S. Krauss, Y. Kawaoka, R.G. Webster, Virology **213**, 223–230 (1995)
32. I. Donatelli, L. Campitelli, L. Di Trani, S. Puzelli, L. Selli, A. Fioretti, D.J. Alexander, M. Tollis, S. Krauss, R.G. Webster, J. Gen. Virol. **82**, 623–630 (2001)
33. C. Abolnik, Avian Dis. **51**, 873–879 (2007)
34. M.L. Perdue, M. Garcia, J. Beck, M. Brugh, D.E. Swayne, Virus Genes **12**, 77–84 (1996)
35. H.M. Kang, O.M. Jeong, M.C. Kim, J.S. Kwon, M.R. Paek, J.G. Choi, E.K. Lee, Y.J. Kim, J.H. Kwon, Y.J. Lee, J. Wildl. Dis. **46**, 878–888 (2010)
36. H.R. Kim, C.K. Park, J.K. Oem, Y.C. Bae, J.G. Choi, O.S. Lee, Y.J. Lee, J. Gen. Virol. **91**, 1978–1983 (2010)
37. J.H. Lee, P.N.Q. Pascua, M.S. Song, Y.H. Baek, C.J. Kim, H.W. Choi, M.H. Sung, R.J. Webby, R.G. Webster, H. Poo, Y.K. Choi, J. Virol. **83**, 4205–4215 (2009)