Reintroduction of H5N1 highly pathogenic avian influenza virus by migratory water birds, causing poultry outbreaks in 2010-2011 winter season in Japan

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Key words: avian influenza, H5N1, surveillance, migratory water birds

Running head: Characterization of H5N1 isolates in Japan
Abstract

H5N1 highly pathogenic avian influenza virus (HPAIV) was reintroduced and caused outbreaks in chickens in 2010-2011 winter season in Japan, that had been free from highly pathogenic avian influenza (HPAI) since 2007 when HPAI outbreaks occurred and were controlled. On October 14, 2010 at Lake Ohnuma, Wakkanai, the northernmost part of Hokkaido, Japan, H5N1 HPAIVs were isolated from fecal samples of ducks flying from their nesting lakes in Siberia. Since then, in Japan, H5N1 HPAIVs have been isolated from 63 wild birds in 17 prefectures and caused HPAI outbreaks in 24 chicken farms in 9 prefectures by the end of March in 2011. Each of these isolates was genetically closely related to the HPAIV isolates at Lake Ohnuma, and those in China, Mongolia, Russia, and Korea, belonging to genetic clade 2.3.2.1. In addition, these isolates were genetically classified into 3 groups, suggesting that the viruses were transmitted by migratory water birds through at least 3 different routes from their northern territory to Japan. These isolates were antigenic variants, which is consistent with the selection in poultry under the immunological pressure induced by vaccination. To prevent the perpetuation of viruses in the lakes where water birds nest in summer in Siberia, prompt eradication of HPAIVs in poultry is urgently needed in Asian countries where the HPAI has not been controlled.
Avian influenza caused by infection with H5N1 highly pathogenic avian influenza virus (HPAIV) has spread in poultry in more than 60 countries in Eurasia and Africa since 1996, when the first outbreak occurred at a goose farm in Guangdong province in China (Smith et al., 2006; Xu et al., 1999). H5N1 HPAIV infections have become endemic in several countries and cause accidental transmissions to humans. H5N1 viruses are thus now recognized as one of the most likely candidates for the next pandemic (Li et al., 2004; Peiris et al., 2007). The widespread presence of H5N1 HPAIVs in poultry, especially in domestic ducks reared in free range, has inevitably resulted in the water-borne transmission of viruses to wild bird populations since domestic ducks and geese infected with HPAIV shed progeny viruses with feces into ponds at farms, where migratory water birds visit. In the past, such infections had been restricted to wild birds found dead in the vicinity of infected poultry farms, but it is now a concern that infections in wild birds in which HPAIV has caused mild clinical signs (e.g., ducks) could result in the spread of viruses to large areas (Kim et al., 2009; Smith et al., 2009). Infection with HPAIVs in many wild bird species at 2 water bird parks in Hong Kong was reported in 2002 (Ellis et al., 2004) and further, more significant outbreaks in wild water birds occurred at Lake Qinghai in Western China, and Khunt and Erkheil Lakes in Mongolia in 2005 (Chen et al., 2005; Sakoda et al., 2010). H5N1 HPAIV infections in poultry and wild birds have now spread in Asia, Europe, and Africa, and it has been suggested that the H5N1 virus could spread by migratory water birds to the west and south, since genetically closely related H5N1
viruses (clade 2.2) have been isolated in several countries since 2005 (Monné et al., 2008; Salzberg et al., 2007; Starick et al., 2008).

In Japan, the outbreaks caused by H5N1 HPAlVs occurred in chicken farms in 2004 (Mase et al., 2005) and 2007. The H5N1 HPAlV isolates in 2004 and 2007 were genetically classified into clade 2.5 and 2.2, respectively. Both outbreaks were controlled by the culling of chickens of the farms where the outbreaks occurred (4 farms in each year), intensive surveillance, and improved biosecurity measures. In addition, the H5N1 HPAlVs were isolated from the jungle crows, mountain hawk eagle, and whooper swans in 2004, 2007, and 2008, respectively (Shivakoti et al., 2010; Tanimura et al., 2006; Uchida et al., 2008). Since then, it was confirmed that Japan was free from HPAIV infection in poultry and wild birds by intensive surveillance.

H5N1 viruses of clade 2.3.2 were first isolated from ducks, geese and other mammals in China and Vietnam in 2005 (Chen et al., 2006; Robertson et al., 2006). In intensive surveillance studies in China, viruses belonging to clade 2.3.2, have been characterized as the dominant isolates in poultry and wild birds (Ellis et al., 2009; Jiang et al., 2010; Kou et al., 2009; Smith et al., 2009). In the updated unified nomenclature of H5 HPAlVs, recent H5N1 isolates belonging to the clade 2.3.2 were defined as clade 2.3.2.1 (WHO/OIE/FAO H5N1 Evolution Working Group, 2011). H5N1 HPAlVs of clade 2.3.2.1 were isolated from migratory water birds in Japan in 2008, in China in 2009, in Mongolia in 2009 and 2010, in Russia in 2009 and 2010, and in Korea in 2010 and 2011 (Kwon et al., 2011; Li et al., 2011; Sakoda et al., 2010; Sharshov et al., 2010; Uchida et al., 2008). In addition, the
infections of chickens and wild birds with HPAIVs belonging to clade 2.3.2.1 have now spread to 
Europe (Reid et al., 2011). These H5N1 HPAIVs were isolated from migratory water birds only on 
the way back to their northern territory, and not from those flying to the south from their nesting 
lakes in Siberia in autumn, suggesting that H5N1 HPAIVs had not dominantly perpetuated at their 
nesting lakes in Siberia until 2009 (Sakoda et al., 2010; Yamamoto et al., 2011).

On October 14, 2010 at Lake Ohnuma, Wakkanai, the northernmost part of Hokkaido, Japan,
H5N1 HPAIVs were isolated from fecal samples from ducks flying from their nesting lakes in Siberia 
(Kajihara et al., 2011). Since then, in Japan, H5N1 HPAIVs have been isolated from 63 wild birds 
and caused HPAI outbreaks in 24 chicken farms by the end of March. The aim of the present study 
is to characterize genetically and antigenically H5N1 viruses isolated from wild birds and chickens 
in Japan.

RESULTS

Isolation and identification of H5N1 HPAIVs from wild birds and chickens

In the intensive surveillance of HPAIV infection in poultry and wild birds, H5N1 HPAIV had not 
been isolated from migratory water birds that flew from their nesting lakes in Siberia to Japan until 
the 2009-2010 winter season (data not shown). In the 2010-2011 winter season, 5,591 dead wild 
birds of about 100 species were found in Japan. After the isolation of H5N1 HPAIVs from fecal 
samples of ducks at Lake Ohnuma, Hokkaido (Kajihara et al., 2011), H5N1 viruses were isolated
from 63 dead wild birds (63 isolates) and chickens of 24 farms (24 isolates) in Japan (Fig. 1b and Table 1). The multiple basic amino acids (RERRRKR/G), which is a marker of HPAIVs (OIE, 2011), was found at the cleavage site of the deduced amino acid sequence of the hemagglutinin (HA) of all 87 isolates. The pathogenicity of the representative 4 isolates, A/duck/Fukushima/2/2011 (H5N1), A/whooper swan/Hokkaido/4/2011 (H5N1), A/peregrine falcon/Tochigi/15/2011 (H5N1), and A/peregrine falcon/Aomori/7/2011 (H5N1) to chickens was evaluated with intravenous pathogenicity index (IVPI) test. All chickens inoculated with each virus died within 3 days post-inoculation and IVPI scores were from 2.80 to 2.98, being categorized as HPAIV in chickens. The nucleotide sequences of the representative H5N1 isolates obtained in the present study have been registered in GenBank/EMBL/DDBJ (Supplementary Table S1).

**Phylogenetic analysis of the H5N1 isolates**

For the phylogenetic analysis of HA genes, 30 isolates were selected from 63 isolates of wild birds and 3 isolates were also selected from 24 isolates of chickens. The HA genes of the representative 33 H5N1 isolates were analyzed by the neighbor-joining method along with those of other HPAIVs recently isolated in Asia (Fig. 2a and 2b). The HA genes of the isolates in the 2010-2011 winter season in Japan were closely related to the isolates from poultry or wild birds in China, Mongolia, Russia and Korea in 2009-2011, and were classified into clade 2.3.2.1. These isolates in Japan were divided into 3 groups (A, B, and C) based on the results of phylogenetic
analysis (Fig. 2b and Table 1). This classification by neighbor-joining method was supported by the analyses using maximum likelihood and most parsimony methods with 1,000 bootstrap replicates (data not shown). In particular, A/duck/Hokkaido/WZ83/2010 (H5N1), the first isolate at Lake Ohnuma, Wakkanai, Hokkaido, in October 2010, indicated with asterisk in Fig. 2b, was classified into group C, not group A containing subsequent isolates in Hokkaido (A/pintail/Hokkaido/1/2011, A/greater scaup/Hokkaido/2/2011, A/whooper swan/Hokkaido/3/2011, A/whooper swan/Hokkaido/4/2011, A/whooper swan/Hokkaido/6/2011, A/whooper swan/Hokkaido/13-21/2011, A/whooper swan/Hokkaido/13-27/2011, A/greater scaup/Hokkaido/28/2011, A/whooper swan/Hokkaido/A13/2011) and Fukushima (A/tufted duck/Fukushima/2/2011, A/tufted duck/Fukushima/4/2011, A/tufted duck/Fukushima/5/2011, A/tufted duck/Fukushima/7/2011, A/tufted duck/Fukushima/16/2011, A/tundra swan/Fukushima/207/2011). All occurrences in Hokkaido after January 2011 were only in the eastern Kushiro area, 350 km southeast from Lake Ohnuma, Wakkanai (Fig. 1b). The cases in the Kushiro area in Hokkaido started in mid-January 2011, and ended in mid-February 2011 (Table 1). The isolates from wild birds in this area were genetically closely related to each other and classified into group A (Fig. 2b). In the group B, all viruses were isolated only from western areas (Aichi, Kyoto, Hyogo, Tokushima, and Shimane). In the group C, viruses were isolated from whole of country (Hokkaido, Aomori, Tochigi, Aichi, Mie, Tottori, Yamaguchi, Kochi, Oita, Nagsaki, Miyazaki, and Kagoshima). In addition, A/mandarin duck/Kochi/3901C005/2011 (H5N1) isolated in Kochi Prefecture, in southwestern Japan, belonging to
group C, had the highest nucleotide identity of the HA gene with A/mallard duck/Korea/W401/2011 (H5N1) and A/mandarin duck/Korea/K10-515/2011 (H5N1) isolated in Korea in the 2010-2011 winter season (Kwon et al., 2011).

To assess the genetic relationship of the HPAIVs in gene segments other than the HA, the nucleotide sequences of the representative 30 H5N1 isolates were analyzed and compared with those of other H5N1 HPAIVs (Supplementary Fig. S1 - S7). These viruses are the isolates from wild birds and were used for the phylogenetic tree analysis of HA gene. Genes of these isolates were closely related to each other, and no genetic reassortment with other previous HPAIVs has been identified.

Each of the PB2, PB1, NP, NA, and M genes of the isolates was divided into 3 genetic groups, corresponding to the classification of the HA genes (group A, B, and C), although a few isolates were not divided into these groups (Supplementary Fig. S1 - S5). Because the sequence identities of PA and NS genes were so high that the genes of these isolates were not classified completely into groups A, B, and C (Supplementary Fig. S6 - S7).

**Antigenic analysis of the HA of the H5N1 HPAIV isolates**

The HAs of H5N1 isolates were antigenically analyzed using a panel of monoclonal antibodies (MAbs) recognizing six different epitopes on the HA of A/duck/Pennsylvania/10218/84 (H5N2) (Okamatsu et al., 2010; Soda et al., 2008; Yamamoto et al., 2011) (Table 2). Each of the non-pathogenic avian influenza viruses (NPAIVs) isolated from migratory ducks in Mongolia and
Hokkaido in 2000-2010 bound to all MAbs used in the present study. Each of the H5N1 HPAIVs isolates before 2005, A/Hong Kong/483/1997 (H5N1), A/Vietnam/1194/2004 (H5N1), A/chicken/Yamaguchi/7/2004 (H5N1), and A/whooper swan/Mongolia/3/2005 (H5N1) bound to most MAbs; however, each of the H5N1 viruses belonging to genetic clade 2.3.2.1, including 2 strains isolated in the present study and A/duck/Hokkaido/WZ83/2010 (H5N1) isolated at lake Ohnuma, Wakkanai, bound only to MAb D101/1.

These H5N1 isolates were also antigenically analyzed using hyperimmunized chicken antisera to A/mallard/Hokkaido/24/2009 (H5N1) and A/whooper swan/Hokkaido/1/2008 (H5N1) (Table 2). A/mallard/Hokkaido/24/2009 (H5N1) was isolated from fecal sample and the antigenicity and pathogenicity of this isolate in chickens were similar to those of other H5 NPAIVs isolated from migratory ducks (Yamamoto et al., 2011). The reactivity of the present H5N1 isolates in Japan with the antiserum to A/mallard/Hokkaido/24/2009 (H5N1) was quite low. In contrast, the reactivity of these H5N1 isolates with antiserum to A/whooper swan/Hokkaido/1/2008 (H5N1) was comparatively high. These results indicate that the HAs of H5N1 isolates in the 2010-2011 winter season in Japan are antigenically distinct from H5 NPAIVs and HPAIVs isolated before 2005.

**DISCUSSION**

In October 2010, H5N1 viruses were isolated from fecal samples of ducks at Lake Ohnuma, Wakkanai, Hokkaido on their way to the south from their nesting lakes in Siberia (Kajihara et al.,...
Since then, nationwide H5N1 HPAIV infections in wild birds and chickens have occurred in Japan, and 63 and 24 isolates were identified from wild birds and chickens, respectively. The present results indicate that the viruses isolated from wild birds and chickens from November 2010 onward were genetically related to the isolates from migratory ducks at Lake Ohnuma, Wakkanai in October 2010. In Hokkaido, H5N1 viruses were isolated in two areas, Wakkanai and Kushiro (Fig. 1b). A/duck/Hokkaido/WZ83/2010 (H5N1), the first isolate at Lake Ohnuma, Wakkanai, was identified as a member of genetic group C, not group A containing subsequent isolates in Kushiro in January and February 2011. Based on the genetic analysis, A/duck/Hokkaido/WZ83/2010 (H5N1) was closely related to A/tundra swan/Tottori/12-002/2010 (H5N1) belonging to the group C. The isolates of group C were detected in the whole of country and some isolates of group C had the highest nucleotide identity to that from wild ducks in Korea (Kwon et al., 2011). By contrast, the isolates of group B were detected only in the western area. Wild water birds start migration from their nesting lakes in the northern territory to the south in the middle of August. The migratory routes of water birds are from Siberia to northern Japan via the Kamchatka Peninsula or Sakhalin Island, and to southern Japan via the Korean Peninsula or the coast of northeastern China (Fig 1a).

Our results indicate that the viruses circulating in different populations of wild migratory birds at their nesting lakes in Siberia in summer were transmitted through at least 3 different routes via China, Korea or Russia to Japan in the 2010-2011 winter season. Then, further virus spread occurred in wild birds at the resting lakes of birds in Japan by water-borne transmission or
predation of carcass. Taken together, our results raise the possibility that H5N1 HPAIVs
perpetuated at the nesting lakes in Siberia before the migration of water birds to Japan.

Concerning the origin of these H5N1 viruses, the HA genes of isolates from chickens and wild
birds in China (Jiang et al., 2010; Li et al., 2011) and from wild birds in Mongolia and Russia in 2009
and 2010 (Sakoda et al., 2010; Shashov et al., 2010) were closely related to those of the present
isolates in Japan. The isolates in Laos in 2010 were recently released in the public database
(accession No. CY098351), although epidemiological information is not available. The season of
isolation of these viruses from wild birds in China, Mongolia, and Russia in 2009 was May to July,
the period when migratory water birds return to their nesting lakes in Siberia. Since Japan and
Mongolia are located on the flyways of migratory water birds that flew from their nesting lakes in
Siberia to the south in autumn, intensive surveillance of avian influenza has been performed in
Hokkaido, Japan, and Mongolia every year since 1996. No HPAIV was found in a total of 634 virus
isolates from 13,740 fecal samples of migratory water birds until 2009 (Sakoda et al., 2010;
Yamamoto et al., 2011). These results suggest that the origin of the viruses isolated from wild birds
in China, Mongolia, and Russia in 2009 was poultry in China, and these viruses did not perpetuate
at their nesting areas in Siberia until 2009. The isolation of H5N1 HPAIVs in 2010 spring in
Mongolia and Russia demonstrates that virus spread from poultry to wild birds occurred again in
China and H5N1 HPAIVs circulated in wild water birds since last summer at their nesting lakes in
Siberia. These viruses have been maintained in wild migratory bird populations and were brought
to Japan in the 2010-2011 winter season. To clarify whether H5N1 HPAIV has dominantly
perpetuated at their nesting lakes in Siberia and viruses are brought by migratory birds from
Siberia to the south in autumn, intensive surveillance of avian influenza in migratory birds should
be strengthened.

HPAIVs are not under immunological selection pressure in the non-vaccinated chicken
population since HPAIV causes acute infection and death in chickens. The generation of escape
mutants against H5 HPAIV was first observed in the follow-up phase of H5N2 HPAIV outbreaks in
Mexico in the 1990s (Lee et al., 2004). Since vaccine use for poultry has increased in several
counties, antigenic variants have been selected in H5N1 HPAIVs under immunological selection
pressure (Cattoli et al., 2011; Chen, 2009; Grund et al., 2011). The present results support the
findings that H5N1 viruses belonging to clade 2.3.2.1 were antigenically distinct from other HPAIVs
and NPAIVs of H5 subtype (Okamatsu et al., 2010; Smith et al., 2009). The vaccination was applied
based on the optimistic expectation to prevent H5N1 influenza virus infection in poultry and
humans; however, several countries using vaccines against H5 HPAIV could not eliminate viruses
yet in poultry because the efficacy of vaccine against HPAI is limited to suppress virus replication,
and does not confer the immunity to prevent infection with the virus. It is reasonable to argue that
vaccination of poultry results in the selection of antigenic variants and the vaccine does not confer
immunity against antigenic variants for humans and animals. To stop the infection with H5 HPAIV
in poultry, thorough culling of infected birds must be carried out in the world.
In the 2010-2011 winter season in Japan, outbreaks of H5N1 HPAIV infection in chicken farms were sporadic, except in Miyazaki Prefecture (13 cases), although a large number of infections in wild birds occurred and the natural environment was contaminated with H5N1 HPAIVs all over the country. In Japan, each of the outbreaks in poultry was controlled by culling, intensive surveillance, improved biosecurity measures, and compensation, without the use of vaccine, and ended in March 2011. H5N1 HPAIV strains have persisted throughout the world for more than 15 years, and antigenic variants have been selected because some countries use vaccines for the control of HPAIV infection. In the chickens vaccinated against HPAIV, it is hardly to find infected ones because they do not show clinical signs, in spite of shedding of viruses. As a result, HPAIV returned to migratory water birds from domestic poultry, and many feral water birds died on the way back to their northern territory in Siberia in spring. Some migratory water birds infected with the virus must have returned to their nesting lakes in Siberia, then disseminate the virus to other birds though water-born transmission at their nesting lakes. To prevent the perpetuation of HPAIVs among migratory water birds at their nesting lakes in Siberia, HPAIVs should be contained within poultry in Asia. We, thus, strongly propose that a stamping-out strategy is the only way to achieve prompt eradication of H5N1 HPAIV and that vaccination may be an optional tool for the control of HPAI in addition to the stamping-out policy. Otherwise, disasters will occur every year throughout Asian countries.
METHODS

Viruses. The H5N1 viruses isolated in the present study and reference H5 viruses shown in Table 2 were propagated in 10-day-old embryonated chicken eggs. As reference strains, H5 NPAIVs isolated from fecal material of migratory ducks (Yamamoto et al., 2011) and H5N1 HPAIVs shown in Table 2 (Kajihara et al., 2011; Mase et al., 2005; Muramoto et al., 2006; Okamatsu et al., 2010; Sakoda et al., 2010; Suarez et al., 1998) were used for antigenic analyses.

Isolation and identification of viruses. Virus isolation has been carried out from fecal samples, tracheal and cloacal swabs, or homogenates of the tissues of wild birds and chickens throughout a year. Fecal samples were mixed with the transport medium containing minimum essential medium (Nissui, Japan), 10,000 U/ml penicillin G (Meiji Seika, Japan), 10 mg/ml streptomycin (Meiji Seika), 0.3 mg/ml gentamicin (Merck, USA), 250 U/ml nystatin (Sigma, USA), and 0.5% bovine serum albumin fraction V (Roche, Switzerland) to yield a 10–20% suspension. Tracheal and cloacal swabs were mixed with 2ml of transport medium. Organ tissue was homogenized with transport medium to yield 10% suspension. Samples from wild birds and chickens were inoculated into the allantoic cavities of 10-day-old embryonated chicken eggs and subtypes of the HA and NA of influenza virus isolates were identified by hemagglutination-inhibition (HI) and neuraminidase-inhibition tests, respectively, according to the standard protocol (OIE, 2011).

H5N1 HPAIVs were isolated from 17 species of dead or diseased wild birds, whooper swans
(Cygnus cygnus), greater scaups (Aythya marila), pintail (Anas acuta), peregrine falcons (Falco peregrinus), tufted ducks (Aythya fuligula), mute swans (Cygnus olor), common pochards (Aythya ferina), little grebes (Tachybaptus ruficollis), great crested grebes (Podiceps cristatus), tundra swans (Cygnus columbianus), black-headed gull (Larus ridibundus), black swan (Cygnus atratus), ural owl (Strix uralensis), mandarin ducks (Aix galericulata), grey heron (Ardea cinerea), hooded cranes (Grus monacha), and goshawk (Accipiter gentilis), found at the waterside of their resting areas and the gardens of private houses in November 2010 - March 2011 (Table 1).

Experimental infection of chickens with H5N1 isolates. To assess the pathogenicity of the representative H5N1 virus isolates, A/duck/Fukushima/2/2011 (H5N1), A/whooper swan/Hokkaido/4/2011 (H5N1), A/peregrine falcon/Tochigi/15/2011 (H5N1), and A/peregrine falcon/Aomori/7/2011 (H5N1), were inoculated intravenously into 4- to 6-week-old chickens (Gallus gallus) for the IVPI test according to the standard protocol (OIE, 2011). Each bird was housed in a self-contained isolator unit (Tokiwa Kagaku, Japan) at a BSL-3 facility at Hokkaido University, Japan.

Sequencing and phylogenetic analysis. For the genetic analysis, 30 isolates were selected from 63 isolates of wild birds and 3 isolates were also selected from 24 isolates of chickens. Viral RNA was extracted from the allantoic fluid of embryonated chicken eggs by TRIZOL LS Reagent (Invitrogen,
USA) and reverse-transcribed with the Uni12 primer (Hoffmann et al., 2001) and M-MLV Reverse Transcriptase (Invitrogen). The full-length or partial sequence of each gene segment was amplified by polymerase chain reaction with gene-specific primer sets reported previously (Hoffmann et al., 2001) or designed exclusively in the present study. The sequences of primers designed in the present study are as follows: PB2-826F: GTT AG GAG AG CA A C AGT A T CA G, PB2-2135R: TC AT T GAT GCTCAATGCCG, PB1-547F: AC AC A TT TC CA GAG AA AGAG, PB1-2128R: TCC ACCATGCTAGAAATCC, PA-38F: GTGC GACA ATGCTTCAATCC, PA-1372R: CCTGCAATGGGATACCTCCGC, NP-57F: TGGA AA CTGGTG GAGAAACGC, NP-1456R: TTGTCTCCGAAGAAATAAGA, M-19F: GTCG AAAC GTACGTTCTCTC, M-853R: GAATCC ACAATATCAAGTGCAAG, and NS-848R: TCA TTAATAAGCTGG AACG. Direct sequencing of each gene segment was performed using an auto sequencer, 3130 and 3500 Genetic Analyzer (Applied Biosystems, USA). To assess the genetic relationship among influenza virus isolates, the nucleotides 34-1,019 (986 bp) of HA, 197-1,206 (1,010 bp) of NA, 1,017-1,929 (913 bp) of PB2, 1,064-1,657 (594 bp) of PB1, 269-1,218 (950 bp) of PA, 760-1,329 (570 bp) of NP, 97-771 (675 bp) of M, and 73-750 (678 bp) of NS of isolates in the present study were compared with those of other recent H5N1 isolates in Asia. For the NA and internal genes, reference strains of each genotype according to the previous report (Duan et al., 2008) were included. Phylogenetic trees were constructed by the neighbor-joining method (Saitou & Nei, 1987) by MEGA 5 software (http://www.megasoftware.net/).
Antigenic analysis. The antigenic properties of the representative H5 viruses, A/duck/Hokkaido/WZ83/2010 (H5N1), A/whooper swan/Hokkaido/4/2011 (H5N1), A/peregrine falcon/Aomori/7/2011 (H5N1), were compared with those of the reference H5 viruses by the fluorescent antibody method using MAbs against H5 HA (Soda et al., 2008). MDCK cells infected with H5 influenza viruses were fixed with cold 100% acetone at 8 hours post-inoculation. The reactivity patterns of the H5 viruses with MAbs were investigated with a FITC-conjugated goat IgG to mouse IgG (MP Biomedicals, USA) by a fluorescence microscope, Axiovert 200 (Carl Zeiss, Germany).

The antigenic properties of the representative H5 viruses were also assessed using hyperimmunized chicken antisera against A/mallard/Hokkaido/24/2009 (H5N1) and A/whooper swan/Hokkaido/1/2008 (H5N1) by HI test according to the standard protocol (OIE, 2011). HI titers were expressed as the reciprocals of the highest serum dilutions that showed complete HI.

ACKNOWLEDGEMENTS

We deeply appreciate the kind cooperation of the Ministry of Environment and Ministry of Agriculture, Forestry and Fisheries, Government of Japan. We also thank Ms. M. Jizou, Ms. M. Endo, and Ms. Y. Sato of Hokkaido University for their technical support. The present work was supported in part by the J-GRID: the Japan Initiative for Global Research Network on Infectious
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Figure legends

Fig. 1 H5N1 HPAIV infections in wild birds and chickens in the 2010-2011 winter season in Japan.

(a) Geographical location of Japan in Asia and migration routes of wild water birds from Siberia in autumn. (b) On October 14, 2010 at Lake Ohnuma, Wakkanai, Hokkaido, Japan (denoted by red star), H5N1 HPAIVs were isolated from fecal samples from ducks that had flown from their nesting lakes in Siberia (Kajihara et al., 2011). H5N1 HPAIVs had been isolated from 63 wild birds in 17 prefectures (denoted by red circles) and chickens of 24 farms in 9 prefectures (denoted by blue circles) by the end of March 2011. The occurrences at different geographical location were indicated by star or circles, and the subsequent cases at the same place were omitted.

Fig. 2 Phylogenetic trees of HA genes of the isolates in the 2010-2011 winter season in Japan. (a)

Phylogenetic tree of H5 avian influenza viruses. The unified nomenclature of the A/goose/Guangdong/1/1996 lineage of Eurasian HPAIVs was based on the homology of HA gene and classified into 10 district clades (clade 0-9) containing second (or third) order clades proposed by the WHO/OIE/FAO H5N1 Evolution Working Group (2008; 2009). Recently, new classification was proposed by the same group (2011) and 2.3.2.1 is one of the new nomenclature system. The H5N1 HPAIVs isolated in this study were classified into clade 2.3.2.1 with other recent isolates in Asia from 2007 onward. A/mallard/Hokkaido/24/09 (H5N1) is indicated as representative strain of NPAIV isolated from water birds and its HA gene was classified into Eurasian lineage (Yamamoto et
al., 2011). HA genes of A/chicken/Pennsylvania/1/1983 (H5N2) and A/chicken/Ibaraki/1/2005 (H5N2) belong to the North American lineage. (b) Phylogenetic trees of HA genes of H5N1 HPAIVs including the isolates in the 2010-2011 winter season in Japan. To assess genetic relationships among H5 avian influenza virus isolates, nucleotide sequences of HA genes of each isolate in the present study were compared with those of recent isolates in Asia in 2007-2011, belonging to genetic clade 2.3.2.1. Phylogenetic trees were constructed by the neighbor-joining method and bootstrap testing (n = 1000). Phylogenetic trees were rooted to A/whooper swan/Hokkaido/1/2008 (H5N1).

The HA genes of the recent isolates in this study (highlighted) was divided into 3 genetic groups (A, B, and C). A/duck/Hokkaido/WZ83/2010, H5N1 HPAIV isolated from fecal samples on October 14, 2010 at Lake Ohnuma, Hokkaido, Japan (Kajihara et al., 2011) was indicated with an asterisk. The isolates in Korea, Russia, Mongolia, China, Laos, and Vietnam in 2007-2011 were underlined.

Horizontal distances are proportional to the minimum number of nucleotide differences required to join nodes and sequences. HA and NA subtypes were left out for the names of H5N1 viruses.

Abbreviation: Ck (chicken).
Fig. 2 Sakoda et al.
<table>
<thead>
<tr>
<th>Areas</th>
<th>Prefectures</th>
<th>Date of reports</th>
<th>Species of birds †</th>
<th>Genetic subgroup of representative isolates ‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Honshu</td>
<td>Aomori</td>
<td>Mar. 10 (2011)</td>
<td>Peregrine falcon (1)</td>
<td>C</td>
</tr>
<tr>
<td></td>
<td>Fukushima</td>
<td>Jan. 4, 5, 7, 10, 23, Feb. 10 (2011)</td>
<td>Tufted duck (5), Tundra swan (1)</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td>Chiba</td>
<td>Mar. 12, 16 (2011)</td>
<td>Chicken (2)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Toyama</td>
<td>Dec. 16 (2010)</td>
<td>Mute swan (1)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Mie</td>
<td>Feb. 15, 26 (2011)</td>
<td>Chicken (2)</td>
<td>C</td>
</tr>
<tr>
<td></td>
<td>Wakayama</td>
<td>Feb. 15 (2011)</td>
<td>Chicken (1)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Kyoto</td>
<td>Feb. 16 (2011)</td>
<td>Peregrine falcon (1)</td>
<td>B</td>
</tr>
<tr>
<td></td>
<td>Nara</td>
<td>Feb. 28 (2011)</td>
<td>Chicken (1)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Hyogo</td>
<td>Jan. 12, 25, Feb. 11, 22 (2011)</td>
<td>Common pochard (1), Little grebe (1), Mute swan (1), Great crested grebe (1)</td>
<td>B</td>
</tr>
<tr>
<td></td>
<td>Nov. 29 (2010)</td>
<td>Chicken (1)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Yamaguchi</td>
<td>Feb. 6, 9 (2011)</td>
<td>Tufted duck (1), Black swan (1)</td>
<td>C</td>
</tr>
<tr>
<td>Shikoku</td>
<td>Tokushima</td>
<td>Feb. 8 (2011)</td>
<td>Ural owl (1)</td>
<td>B</td>
</tr>
<tr>
<td></td>
<td>Oita</td>
<td>Feb. 7, 8, 9, 15 (2011)</td>
<td>Mandarin duck (4), Grey heron (1)</td>
<td>C</td>
</tr>
<tr>
<td></td>
<td>Feb. 2 (2011)</td>
<td>Chicken (1)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Miyazaki</td>
<td>Feb. 1, 2, 8, 11, 14, 15, 18 (2011)</td>
<td>Mandarin duck (3), Peregrine falcon (3), Little grebe (1)</td>
<td>C</td>
</tr>
<tr>
<td></td>
<td>Jan. 22, 24, 27, 28, 29, 30, Feb. 1, 4, 5, 6, 7, 17, Mar. 5 (2011)</td>
<td>Chicken (13)</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

* Information about the case in chicken farm is underlined.
† Number of dead wild birds or outbreaks in chicken farm is in parentheses.
‡ Based on the phylogenetic tree of HA gene shown in Fig. 1.
§ Viruses were isolated from fecal sample (Kajihara et al., 2011).
¶ Not tested.
Table 2  Antigenic analyses of H5 influenza viruses

<table>
<thead>
<tr>
<th>Viruses *</th>
<th>Clades</th>
<th>Monoclonal antibodies †</th>
<th>Polyclonal antibodies ‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>I (88)</td>
<td>II (145)</td>
</tr>
<tr>
<td>NPAIV</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dk/Pennsylvania/10218/1984 (H5N2)</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Dk/Mongolia/54/2001 (H5N2)</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Dk/Hokkaido/167/2007 (H5N3)</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Dk/Hokkaido/WZ21/2008 (H5N2)</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Mal/Hokkaido/24/2009 (H5N1)</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Dk/Hokkaido/101/2010 (H5N2)</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>HPAIV</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hong Kong/483/1997 (H5N1)</td>
<td>0</td>
<td>-</td>
<td>+</td>
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<td>Vietnam/1194/2004 (H5N1)</td>
<td>1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Ck/Yamaguchi/7/2004 (H5N1)</td>
<td>2.5</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Ws/Mongolia/3/2005 (H5N1)</td>
<td>2.2</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Ws/Hokkaido/1/2008 (H5N1)</td>
<td>2.3.2.1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ws/Mongolia/6/2009 (H5N1)</td>
<td>2.3.2.1</td>
<td>-</td>
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<tr>
<td>Ws/Mongolia/1/2010 (H5N1)</td>
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<tr>
<td>Dk/Hokkaido/WZ83/2010 (H5N1)</td>
<td>2.3.2.1</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Ws/Hokkaido/4/2011 (H5N1)</td>
<td>2.3.2.1</td>
<td>-</td>
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<tr>
<td>Pf/Aomori/7/2011 (H5N1)</td>
<td>2.3.2.1</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* Viruses indicated in bold were the isolates in 2010-2011 winter season in Japan. Abbreviations: Dk (duck), Mal (mallard), Ck (chicken), Ws (whooper swan), Pf (peregrine falcon).
† Reactivity of monoclonal antibodies against the HA of A/duck/Pennsylvania/10218/1984 (H5N2) to the representative H5 viruses were compared in fluorescent antibody methods. Location of amino acid substitutions in antigenic variants selected in the presence of respective monoclonal antibodies (Soda et al., 2008) is indicated in parentheses.
‡ HI titers of hyperimmunized polyclonal antibodies against representative H5 viruses were measured.