clinically for 14 days. When chickens died or were sacrified, tracheal and cloacal swabs and their tissues (trachea, kidney, colon) were collected. Virus infectivity titers were determined by plaque assays.

Plaque assays: Virus infectivity titers in swabs and tissue samples were determined by plaque assay as described[13]. Briefly, MDCK cells were grown in 6-well tissue culture plates, when monolayer became confluent, ten-fold serial dilutions of swab and tissue samples were prepared in minimal essential medium (MEM) and 200 μl of each dilution was added to each well. After 1 hour adsorption at 37°C the inoculums were removed and the cells were overlaid with Eagle's MEM containing 0.9% Bacto-Agar (Difco). After incubation for 24–48 hrs, second overlay containing neutral red (0.005%) was made and plaques were enumerated after overnight incubation. The limit of virus detection was 10^3 plaque forming units (PFU)/g of tissues or ml of swabs.

Results

Antigenic relatedness among the challenge and vaccine strain viruses

Okamatsu *et al.*, 2010[9] has shown that

Table 1. HI titers of the sera of chickens before (0) and 14 days after challenge

Vaccine strains	#[a]	HI titers with the following antigens on the day post challenge					
		Dk/Vac-1/04		Ws/Mon/05		Ws/Hok/08	
		0	14	0	14	0	14
Vac-1/04	1	640	-[b]	320	-	320	-
	2	1280	-	160	-	160	-
	3	1280	-	160	-	80	-
	4	5120	-	1280	-	80	-
	5	5120	10240	1280	5120	80	640
	6	640	2560	160	1280	160	320
	7	2560	20480	160	5120	40	2560
	8	640	2560	160	1280	80	640
	9	2560	5120	80	1280	40	640
	10	5120	20480	320	640	40	160
rg-Mon/05	11	40	-	80	-	80	-
	12	20	-	40	-	80	-
	13	80	-	640	-	320	-
	14	40	-	320	-	160	-
	15	320	1280	640	2560	640	1280
	16	1280	2560	1280	2560	160	640
	17	640	640	640	640	80	160
	18	1280	2560	1280	5120	320	1280
	19	640	1280	640	1280	320	640
	20	320	1280	640	1280	320	1280

[a]: Chicken number
[b]: no samples tested

antigenicities of the HA of the isolates in 2008 were different from the H5N1 viruses isolated from wild birds and poultry before 2007. These findings indicate that the challenge virus strain Ws/Hok/08 is antigenically different from Dk/Vac-1/04.

Pathogenicity of ΔRRRRK rg-Mon/05 (H5N1)

None of the chickens inoculated intravenously with ΔRRRRK rg-Mon/05 showed clinical signs in the 10 days observation period. The IVPI value of the strain is 0.00 indicating that ΔRRRRK rg-Mon/05 is non-apathogenic for chickens. No virus was recovered from organs of the chickens inoculated with ΔRRRRK rg-Mon/05 except one bird from which organ sample trachea showed $10^{1.75}$ EID_{50}/g.

Potency of vaccines in chickens

Table 1 shows serum HI antibody titers in the vaccinated chickens. The HI antibody titers of the vaccinated chickens showed increase in the antibody titers after 3 weeks of vaccination. Chickens inoculated with the test vaccines were challenged with HPAIV, Ws/Hok/08 on 3 weeks post vaccination. Two weeks after challenge, approximately 2^4 fold of HI antibody titers were increased in all vaccinated chickens to the homologous and heterologus viruses indicating virus replication occurred. In addition, all vaccinated chickens survived after challenge throughout 14 observation days without showing any disease signs, whereas all of the control chickens died within 2 days post challenge (Fig. 1). Viruses were not recovered from swabs and tissue samples of any of the vaccinated chickens after challenge by plaque assay.

Discussion

It is strongly recommended that stamping-out without misuse of vaccine is the best option for the eradication of HPAIV. Vaccination may be an optional tool in cases where the infection spreads widely[1]. Such a vaccine should ideally meet the following criteria: (i) safe for both hosts and the environment; (ii) economically feasible; and (iii) efficacious.

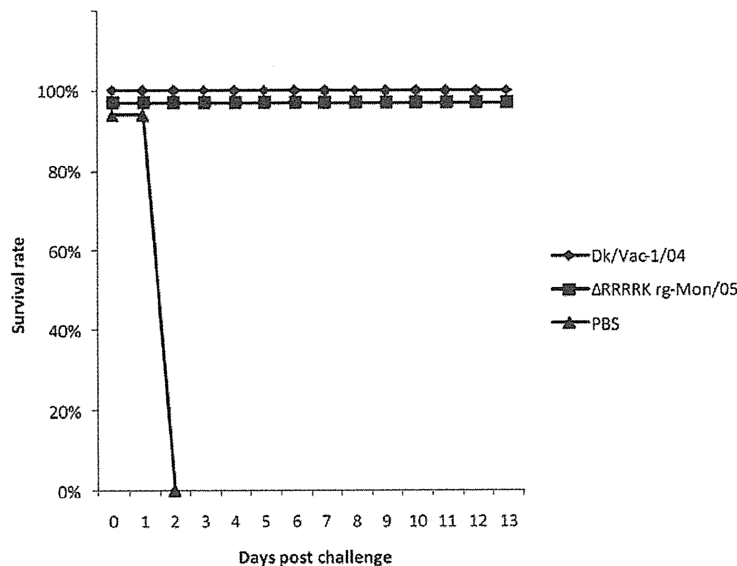

Fig. 1. Survival of chickens after challenge with Ws/Hok/08. Ten 4-week-old chickens in each group were vaccinated with Dk/Vac-1/04, and ΔRRRRK rg-Mon/05 respectively. Three weeks after vaccination, chickens were challenged with Ws/Hok/08 (H5N1) virus. Control chickens (black triangle) were dead within 24 to 48 hr after challenge. Vaccinated chickens were observed for 14 days.

In the present study, both vaccine strains induced sufficient antibody response against the challenge with phylogenetically and antigenically different HPAIV. Previous studies[5,9] have shown that Dk/Vac-1/04 vaccine protect chickens from clinical signs and induced antibodies against homologous and heterologous strains after challenge. In the present study, it was shown that vaccinated chickens with Dk/Vac-1/04 not only induced antibodies against homologous and heterologous strain but conferred protective immunity to chickens against the challenge of antigenically drifted HPAIV. These findings indicate that vaccine prepared from non-pathogenic avian influenza virus from the virus library is efficacious and protect chickens from HPAI. Furthermore, chickens even challenged with higher doses of viral challenge (10^3 CLD_{50}) did not show clinical signs indicating that the vaccines induced sufficient protective immunity in chickens to prevent clinical manifestations.

On the basis of the findings in the present study, inactivated influenza virus vaccine prepared from a non-pathogenic influenza virus strain from the virus library conferred protective immunity against the challenge with antigenically drifted HPAIV. The efficacy of the vaccine was comparable to that prepared from genetically modified HPAIV strain ΔRRRRK rg-Mon/05, which is more antigenically related to the challenge virus strain, in chickens. Therefore, it is proposed that vaccine strain selected from the non-pathogenic influenza virus library is efficacious and safe in protecting chickens from HPAI.

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

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H5N1 highly pathogenic avian influenza virus (HPAIV) was reintroduced and caused outbreaks in chickens in the 2010–2011 winter season in Japan, which had been free from highly pathogenic avian influenza (HPAI) since 2007 when HPAI outbreaks occurred and were controlled. On 14 October 2010 at Lake Ohnuma, Wakkanai, the northernmost part of Hokkaido, Japan, H5N1 HPAIVs were isolated from faecal 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 nine 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 three groups, suggesting that the viruses were transmitted by migratory water birds through at least three different routes from their northern territory to Japan. These isolates were antigenic variants, which is consistent with 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 HPAI has not been controlled.

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Supplementary figures and a supplementary table with the GenBank/EMBL/DDBJ accession numbers of the nucleotide sequences of the representative H5N1 isolates determined in this study are available with the online version of this paper.

INTRODUCTION

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 free-range-reared ducks, has inevitably resulted in the water-borne transmission of viruses to wild-bird populations, as domestic ducks and geese infected with HPAIV shed progeny virus with their faeces into ponds on farms where migratory water birds visit. In the past, such infections were 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 two water-bird parks in Hong Kong was reported in 2002 (Ellis *et al.*, 2004), and more significant outbreaks in wild water birds occurred at Lake Qinghai in western China, and at the Khunt and Erkhel 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, as genetically closely related H5N1 viruses (clade 2.2) have been isolated in several countries since 2005 (Monne *et al.*, 2008; Salzberg *et al.*, 2007; Starick *et al.*, 2008).

In Japan, the outbreaks caused by H5N1 HPAIVs occurred in chicken farms in 2004 (Mase *et al.*, 2005) and 2007. The H5N1 HPAIV isolates in 2004 and 2007 were classified genetically into clades 2.5 and 2.2, respectively. Both outbreaks were controlled by the culling of chickens on the farms where the outbreaks occurred (four farms in each year), intensive surveillance and improved biosecurity measures. In addition, H5N1 HPAIVs were isolated from jungle crows, mountain hawk eagles and whooper swans in 2004, 2007 and 2008, respectively (Shivakoti *et al.*, 2010; Tanimura *et al.*, 2006; Uchida *et al.*, 2008). Since then, it has been 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; Roberton *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 HPAIVs, recent H5N1 isolates belonging to clade 2.3.2 were defined as clade 2.3.2.1 (WHO/OIE/FAO H5N1 Evolution Working Group, 2012). H5N1 HPAIVs 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 only from migratory water birds 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 14 October 2010 at Lake Ohnuma, Wakkanai, in the northernmost part of Hokkaido, Japan, H5N1 HPAIVs were isolated from faecal 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 was to characterize genetically and antigenically the 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, 5591 dead wild birds of ~100 different species were found in Japan. After the isolation of H5N1 HPAIVs from faecal samples of ducks at Lake Ohnuma, Hokkaido (Kajihara *et al.*, 2011), H5N1 viruses were isolated from 63 dead wild birds (63 isolates) and chickens from 24 farms (24 isolates) in Japan (Fig. 1 and Table 1). The multiple basic amino acid sequence (RERRRKR/G), which is a marker of HPAIVs (OIE, 2011), was found at the cleavage site of the deduced amino acid sequence of the haemagglutinin (HA) of all 87 isolates. The pathogenicity of four representative 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 using an intravenous pathogenicity index (IVPI) test. All chickens inoculated with each virus died within 3 days of inoculation, and IVPI scores ranged from 2.80 to 2.98, which were categorized as HPAIV in chickens. The nucleotide sequences of the representative H5N1 isolates obtained in the present study have been registered in GenBank and are given in Supplementary Table S1 (available in JGV Online).

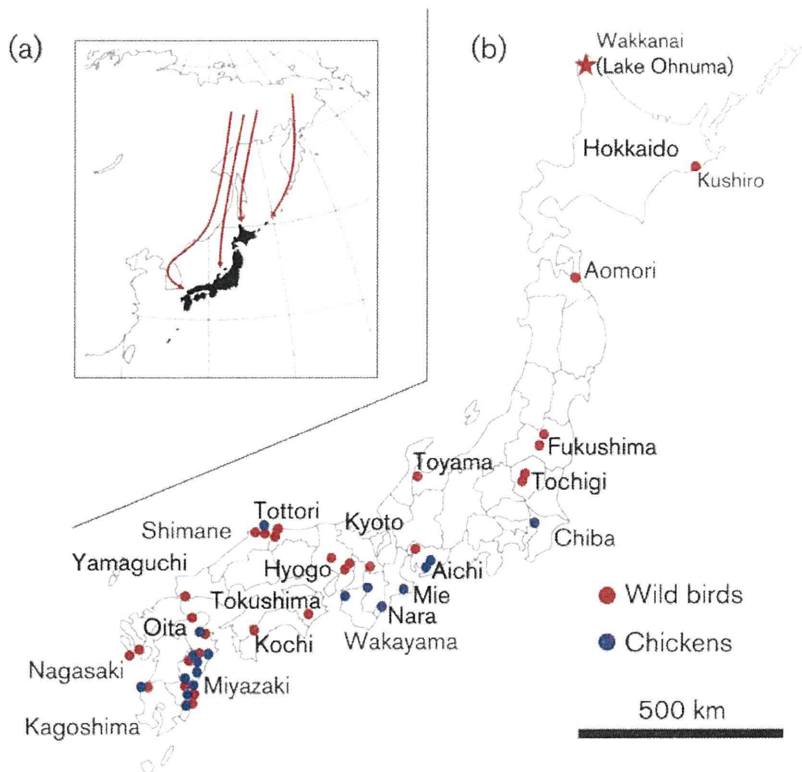

Fig. 1. H5N1 HPAIV infections in wild birds and chickens in the 2010–2011 winter season in Japan. (a) Geographical location of Japan in Asia and migration routes of wild water birds from Siberia in autumn (arrows). (b) On 14 October 2010 at Lake Ohnuma, Wakkanai, Hokkaido, Japan (red star), H5N1 HPAIVs were isolated from faecal samples from ducks that had flown from their nesting lakes in Siberia (Kajihara *et al.*, 2011). H5N1 HPAIVs were isolated from 63 wild birds in 17 prefectures (red circles) and chickens of 24 farms in nine prefectures (blue circles) by the end of March 2011. Occurrences at different geographical location are indicated by the star or circles, and subsequent cases at the same place are omitted.

Phylogenetic analysis of the H5N1 isolates

For the phylogenetic analysis of HA genes, 30 isolates were selected from 63 isolates of wild birds, and three isolates were also selected from 24 isolates of chickens. The HA genes of the representative 33 H5N1 isolates were analysed by the neighbour-joining method along with those of other HPAIVs isolated recently in Asia (Fig. 2a). 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 three groups (A–C) based on the results of phylogenetic analysis (Fig. 2b and Table 1). This classification by the neighbour-joining method was supported by analyses using maximum-likelihood and maximum-parsimony methods with 1000 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 an asterisk in Fig. 2(b), was classified into group C, and not group A containing subsequent isolates from 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 and 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 and A/tundra swan/Fukushima/207/2011). All occurrences in Hokkaido after January 2011 were only in the eastern Kushiro area, 350 km south-east of 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 were classified into group A (Fig. 2b). In group B, all viruses were isolated only from western areas (Aichi, Kyoto, Hyogo, Tokushima and Shimane). In group C, viruses were isolated from the whole of the country (Hokkaido, Aomori, Tochigi, Aichi, Mie, Tottori, Yamaguchi, Kochi, Oita, Nagasaki, Miyazaki and Kagoshima). In addition, A/mandarin duck/Kochi/3901C005/2011 (H5N1) isolated in Kochi Prefecture, in south-western 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 a representative 30 H5N1 isolates were analysed and compared with those of other H5N1 HPAIVs (see Supplementary Figs S1–S7, available in JGV Online). These viruses were isolates from wild birds and were used for the phylogenetic tree analysis of the HA gene. The genes of these isolates were closely related to each other, and no genetic reassortment with other previous HPAIVs was identified. Each of the polymerase subunit (PB2 and PB1), nucleoprotein (NP),

Table 1. Cases of infection with H5N1 HPAIVs in Japan in the 2010–2011 winter season

Information about the cases from chicken farms is underlined. NT, Not tested.

Area	Prefecture	Date of reports	Species of birds*	Genetic subgroup of representative isolates†
Hokkaido	Hokkaido	14 Oct 2010‡, 12, 17, 18, 19 and 28 Jan 2011, 3, 7 and 17 Feb 2011	Duck (2)‡, whooper swan (6), greater scaup (2), pintail (1)	A, C‡
Honshu	Aomori	10 Mar (2011)	Peregrine falcon (1)	C
	Fukushima	4, 5, 7, 10 and 23 Jan 2011, 10 Feb 2011	Tufted duck (5), tundra swan (1)	A
	Tochigi	14 Feb 2011, 25 Mar 2011	Peregrine falcon (1), goshawk (1)	C
	Chiba	12 and 16 Mar 2011	Chicken (2)	NT
	Aichi	17 Feb 2011	Peregrine falcon (1)	B, C
		27 Jan 2011, 14 Feb 2011	Chicken (2)	
	Toyama	16 Dec 2010	Mute swan (1)	NT
	Mie	15 and 26 Feb 2011	Chicken (2)	C
	Wakayama	15 Feb 2011	Chicken (1)	NT
	Kyoto	16 Feb 2011	Peregrine falcon (1)	B
	Nara	28 Feb 2011	Chicken (1)	NT
	Hyogo	12 and 25 Jan 2011, 11 and 22 Feb 2011	Common pochard (1), little grebe (1), mute swan (1), great crested grebe (1)	B
	Tottori	4 Dec 2010, 19 and 24 Jan 2011, 1, 3 and 6 Feb 2011	Tundra swan (1), black-headed gull (1), tufted duck (2), common pochard (1), peregrine falcon (1)	C
	Shimane	14 Jan 2011, 1 and 8 Feb 2011	Tufted duck (4), common pochard (1)	B
		29 Nov 2010	Chicken (1)	
	Yamaguchi	6 and 9 Feb 2011	Tufted duck (1), black swan (1)	C
Shikoku	Tokushima	8 Feb 2011	Ural owl (1)	B
	Kochi	26 Jan 2011	Mandarin duck (1)	C
Kyushu	Nagasaki	31 Jan 2011, 4 and 12 Feb 2011	Mandarin duck (3), peregrine falcon (1)	C
	Oita	7, 8, 9 and 15 Feb 2011	Mandarin duck (4), grey heron (1)	C
		2 Feb 2011	Chicken (1)	
	Miyazaki	1, 2, 8, 11, 14, 15 and 18 Feb 2011	Mandarin duck (3), peregrine falcon (3), little grebe (1)	C
		22, 24, 27, 28, 29 and 30 Jan 2011, 1, 4, 5, 6, 7 and 17 Feb 2011, 5 Mar 2011	Chicken (13)	
	Kagoshima	19, 20, 21 and 24 Dec 2010, 13 Feb 2011	Hooded crane (7)	C
		26 Jan 2011	Chicken (1)	

*The number of dead wild birds or outbreaks in chicken farms is shown in parentheses.
†Based on the phylogenetic tree of the HA gene shown in Fig. 1.
‡Viruses were isolated from faecal samples (Kajihara *et al.*, 2011).

neuraminidase (NA) and matrix (M) genes of the isolates was divided into three genetic groups, corresponding to the classification of the HA genes (groups A–C), although a few isolates were not divided into these groups (Supplementary Figs S1–S5). Because the sequence identities of the polymerase subunit (PA) and non-structural (NS) genes were so high, the genes of these isolates were not classified completely into groups A, B and C (Supplementary Figs S6 and S7).

Antigenic analysis of the HA of the H5N1 HPAIV isolates

The HAs of H5N1 isolates were analysed antigenically using a panel of 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 the mAbs used in the present study. Each of the H5N1 HPAIVs isolated 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 two strains isolated in the present study and A/duck/Hokkaido/WZ83/2010 (H5N1) isolated at Lake Ohnuma, Wakkanai, bound only to mAb D101/1 (Table 2).

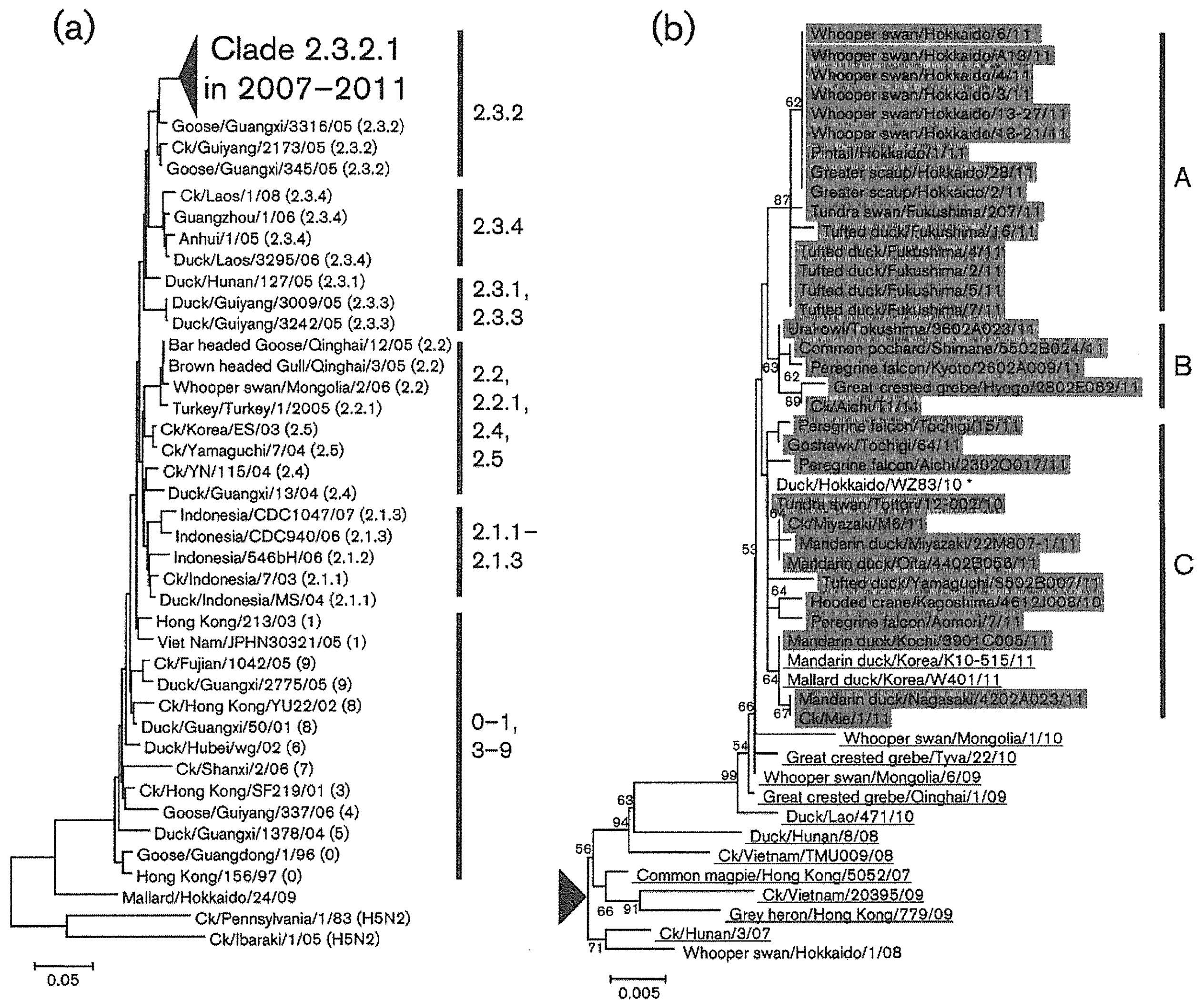

Fig. 2. Phylogenetic trees of HA genes of the isolates in the 2010–2011 winter season in Japan. (a) Phylogenetic tree of H5 avian influenza viruses. The unified nomenclature of the A/goose/Guangdong/1/1996 lineage of Eurasian HPAIVs was based on the homology of the HA gene and classified into ten distinct clades (clades 0–9) containing second-order (or third-order) clades proposed by the WHO/OIE/FAO H5N1 Evolution Working Group (2008, 2009). Recently, a new classification was proposed by the same group (WHO/OIE/FAO H5N1 Evolution Working Group, 2012) and 2.3.2.1 is one of the new nomenclature systems. 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 the Eurasian lineage (Yamamoto *et al.*, 2011). The HA genes of A/chicken/Pennsylvania/1/1983 (H5N2) and A/chicken/Ibaraki/1/2005 (H5N2) belong to the North American lineage. The individual clade of each isolate is shown in parentheses. (b) Phylogenetic trees of the HA genes of H5N1 HPAIVs including the isolates in the 2010–2011 winter season in Japan. To assess the genetic relationships among H5 avian influenza virus isolates, the nucleotide sequences of the HA gene 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 neighbour-joining method and bootstrap testing (1000 replicates). The phylogenetic tree was rooted to A/whooper swan /Hokkaido/1/2008 (H5N1). The HA genes of the recent isolates in this study (shaded) were divided into three genetic groups (A–C). A/duck/Hokkaido/WZ83/2010 (H5N1) HPAIV, isolated from faecal samples on 14 October 2010 at Lake Ohnuma, Hokkaido, Japan (Kajihara *et al.*, 2011), is indicated with an asterisk. Isolates from Korea, Russia, Mongolia, China, Laos and Vietnam in 2007–2011 are underlined. Horizontal distances (bars) are proportional to the minimum number of nucleotide differences required to join nodes and sequences. HA and NA subtypes have been left out of the names of the H5N1 viruses. Ck, Chicken.

Table 2. Antigenic analyses of H5 influenza viruses

Virus*	Clade	mAb†							Polyclonal antibody HI titre‡	
		I (88)	II (145)	III (157)	IV (168)		V (169)	VI (205)	Mal/ Hok/09 (H5N1)	Ws/ Hok/08 (H5N1)
		D101/1	A310/39	64/1	B9/5	B220/1	B59/5	25/2		
NPAIV										
Dk/Pennsylvania/ 10218/1984 (H5N2)	−	+	+	+	+	+	+	+	1280	80
Dk/Mongolia/54/2001 (H5N2)	−	+	+	+	+	+	+	+	640	80
Dk/Hokkaido/167/2007 (H5N3)	−	+	+	+	+	+	+	+	1280	160
Dk/Hokkaido/WZ21/ 2008 (H5N2)	−	+	+	+	+	+	+	+	2560	80
Mal/Hokkaido/24/2009 (H5N1)	−	+	+	+	+	+	+	+	1280	160
Dk/Hokkaido/101/2010 (H5N2)	−	+	+	+	+	+	+	+	640	80
HPAIV										
Hong Kong/483/1997 (H5N1)	0	−	+	+	+	+	+	+	1280	320
Vietnam/1194/2004 (H5N1)	1	+	+	+	+	+	−	+	640	640
Ck/Yamaguchi/7/2004 (H5N1)	2.5	−	+	+	+	+	−	+	1280	1280
Ws/Mongolia/3/2005 (H5N1)	2.2	+	−	+	+	+	−	+	320	640
Ws/Hokkaido/1/2008 (H5N1)	2.3.2.1	+	−	−	−	−	−	−	40	1280
Ws/Mongolia/6/2009 (H5N1)	2.3.2.1	+	−	−	−	−	−	−	80	1280
Ws/Mongolia/1/2010 (H5N1)	2.3.2.1	+	−	−	−	−	−	−	80	640
Dk/Hokkaido/WZ83/ 2010 (H5N1)	**2.3.2.1**	**+**	**−**	**−**	**−**	**−**	**−**	**−**	**40**	**320**
Ws/Hokkaido/4/2011 (H5N1)	**2.3.2.1**	**+**	**−**	**−**	**−**	**−**	**−**	**−**	**40**	**320**
Pf/Aomori/7/2011 (H5N1)	**2.3.2.1**	**+**	**−**	**−**	**−**	**−**	**−**	**−**	**40**	**320**

*Viruses indicated in bold are isolates from the 2010–2011 winter season in Japan. Dk, Duck; Mal, mallard; Ck, chicken; Ws, whooper swan; Pf, peregrine falcon.
†Reactivity of mAbs against the HA of A/duck/Pensylvania/10218/1984 (H5N2) to the representative H5 viruses (I–VI) were compared in fluorescent antibody methods. The location of amino acid substitutions in antigenic variants selected in the presence of the respective mAbs (Soda *et al.*, 2008) is indicated in parentheses.
‡Haemagglutination inhibition (HI) titres of hyperimmunized polyclonal antibodies against representative H5 viruses were measured. Homologous combination between virus and polyclonal antibody is indicated by underlining.

These H5N1 isolates were also analysed antigenically 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 a faecal 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 indicated that the HAs of H5N1 isolates in the 2010–2011 winter season in Japan were

antigenically distinct from H5 NPAIVs and HPAIVs isolated before 2005.

DISCUSSION

In October 2010, H5N1 viruses were isolated from faecal samples of ducks at Lake Ohnuma, Wakkanai, Hokkaido, on their way south from their nesting lakes in Siberia (Kajihara *et al.*, 2011). 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, and 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 group C. The isolates of group C were detected over the whole of the country, and some isolates of group C had the highest nucleotide identity with that of 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 their 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 north-eastern China (Fig. 1a). Our results indicated that the viruses circulating in different populations of wild migratory birds at their nesting lakes in Siberia in summer were transmitted through at least three different routes via China, Korea and Russia to Japan in the 2010–2011 winter season. Further virus spread then occurred in wild birds at the resting lakes of birds in Japan by water-borne transmission or predation of carcasses. Taken together, these results raise the possibility that H5N1 HPAIVs were 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; Sharshov *et al.*, 2010) were closely related to those of the present isolates in Japan. The isolates in Laos in 2010 were released recently in the public database (GenBank 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. As Japan and Mongolia are located on the flyways of migratory water birds that fly 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 faecal samples of migratory water birds until 2009 (Sakoda *et al.*, 2010; Yamamoto *et al.*, 2011). These results suggested that the origin of the viruses isolated from wild birds in China, Mongolia and Russia in 2009 was poultry in China, and that these viruses did not perpetuate at their nesting areas in Siberia until 2009. The isolation of H5N1 HPAIVs in the spring of 2010 in Mongolia and Russia demonstrated that virus spread from poultry to wild birds occurred again in China and that H5N1 HPAIVs have 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 been perpetuated dominantly 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 populations as 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). As 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 of preventing H5N1 influenza virus infection in poultry and humans; however, several countries using vaccines against H5 HPAIV have not yet eliminated viruses in poultry because the efficacy of the vaccine against HPAI is limited to suppress virus replication, and does not confer immunity to prevent infection with the virus. It is reasonable to argue that vaccination of poultry results in the selection of antigenic variants and that the vaccine does not confer immunity against antigenic variants for humans and animals. To stop infection with H5 HPAIV in poultry, thorough culling of infected birds must be carried out worldwide.

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 throughout 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 a 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 hard to find infected birds because they do not show clinical signs, despite shedding of the virus. As a result, HPAIV has returned to migratory water birds from domestic poultry, and many feral water birds have 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 and then disseminated the virus to other birds though water-borne 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. Thus, we strongly recommend 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 the reference H5 viruses shown in Table 2 were propagated in 10-day-old embryonated chicken eggs. As reference strains, H5 NPAIVs isolated from the faecal material of migratory ducks (Yamamoto *et al.*, 2011) and the 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 was carried out from faecal samples, tracheal and cloacal swabs, or homogenates of the tissues of wild birds and chickens throughout the year. Faecal samples were mixed with transport medium containing minimum essential medium (Nissui), 10 000 U penicillin G (Meiji Seika) ml^{-1}, 10 mg streptomycin (Meiji Seika) ml^{-1}, 0.3 mg gentamicin (Merck) ml^{-1}, 250 U nystatin (Sigma) ml^{-1} and 0.5 % BSA fraction V (Roche) to yield a 10–20 % suspension. Tracheal and cloacal swabs were mixed with 2 ml transport medium. Organ tissue was homogenized with transport medium to yield a 10 % suspension. Samples from wild birds and chickens were inoculated into the allantoic cavities of 10-day-old embryonated chicken eggs, and the subtypes of the HA and NA of influenza virus isolates were identified by haemagglutination inhibition (HI) and neuraminidase inhibition tests, respectively, according to a standard protocol (OIE, 2011).

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

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 4–6-week-old chickens (*Gallus gallus*) for an IVPI test according to a standard protocol (OIE, 2011). Each bird was housed in a self-contained isolator unit (Tokiwa Kagaku) at a Biosafety Level 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 three isolates were also selected from 24 isolates of chickens. Viral RNA was extracted from the allantoic fluid of embryonated chicken eggs using TRIzol LS reagent (Invitrogen) and reverse transcribed with the Uni12 primer (Hoffmann *et al.*, 2001) and Moloney murine leukemia virus reverse transcriptase (Invitrogen). The full-length or partial sequence of each gene segment was amplified by PCR 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 were: PB2-826F: 5′-GTTAGGAGAGCAACAGTATCAG-3′, PB2-2135R: 5′-TCATTGATGCTCAATGCCGG-3′, PB1-547F: 5′-ACACATTTCCAGAGAAAGAG-3′, PB1-2128R: 5′-TCCACCATGCTAGAAATCCC-3′, PA-38F: 5′-GTGCGACAATGCTTCAATCC-3′, PA-1372R: 5′-CCTGCAATGGGATACTTCCGC-3′, NP-57F: 5′-TGGAAACTGGTGGAGAACGC-3′, NP-1456R: 5′-TTGTCTCCGAAGAAATAAGA-3′, M-19F: 5′-GTCGAAACGTACGTTCTCTC-3′, M-853R: 5′-GAATCCACAATATCAAGTGCAAG-3′ and NS-848R: 5′-TCATTAAATAAGCTGGAACG-3′. Direct sequencing of each gene segment was performed using a 3130 or 3500 Genetic Analyzer (Applied Biosystems). To assess the genetic relationship among influenza virus isolates, nt 34–1019 (986 bp) of HA, nt 197–1206 (1010 bp) of NA, nt 1017–1929 (913 bp) of PB2, nt 1064–1657 (594 bp) of PB1, nt 269–1218 (950 bp) of PA, nt 760–1329 (570 bp) of NP, nt 97–771 (675 bp) of M and nt 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 a previous report (Duan *et al.*, 2008) were included. Phylogenetic trees were constructed by the neighbour-joining method (Saitou & Nei, 1987) using 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) and A/peregrine falcon/Aomori/7/2011 (H5N1)] were compared with those of the reference H5 viruses by a fluorescent antibody method using mAbs against H5 HA (Soda *et al.*, 2008). Madin–Darby canine kidney cells infected with H5 influenza viruses were fixed with cold 100 % acetone at 8 h post-inoculation. The reactivity patterns of the H5 viruses with mAbs were investigated with an FITC-conjugated goat anti-mouse IgG (MP Biomedicals) using a fluorescence microscope (Axiovert 200; Carl Zeiss).

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 an HI test according to a standard protocol (OIE, 2011). HI titres were expressed as the reciprocals of the highest serum dilutions that showed complete HI.

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1 *Virology*

2 *Full paper*

3 **An H9N2 influenza virus vaccine prepared from a non-pathogenic isolate from a migratory**

4 **duck confers protective immunity in mice against challenge with an H9N2 virus isolated from**

5 **a girl in Hong Kong**

6

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19 *Running head:* A VACCINE AGAINST H9N2 INFLUENZA VIRUS

20

21 **ABSTRACT.** H9N2 influenza viruses circulate in wild birds and poultry in Eurasian countries,
22 and have been isolated from pigs and humans in China. H9N2 viruses isolated from birds, pigs and
23 humans have been classified into three sublineages based on antigenic and genetic features.
24 Chicken antisera to H9N2 viruses of the Korean sublineage reacted with viruses of different
25 sublineages by the hemagglutination-inhibition test. A test vaccine prepared from a non-pathogenic
26 A/duck/Hokkaido/49/1998 (H9N2) strain of the Korean sublineage, obtained from our influenza
27 virus library, induced immunity in mice to reduce the impact of disease caused by the challenge with
28 A/Hong Kong/1073/1999 (H9N2), which is of a different sublineage. The present results indicate
29 that an inactivated whole virus vaccine prepared from a non-pathogenic influenza virus from the
30 library could be used as an emergency vaccine during the early stage of a pandemic caused by H9N2
31 infection.

32

33 KEY WORDS: H9N2 influenza virus, antigenicity, vaccine

34

35 **INTRODUCTION**

36 Avian influenza viruses of various subtypes are circulating in poultry worldwide [1, 18-19, 21,
37 29, 38]. In particular, H9N2 influenza virus is prevailing poultry populations in Eurasian countries
38 [9-11, 24]. Since H9N2 viruses were isolated from quails in Hong Kong in 1988, they have
39 become prevalent in live bird markets and poultry farms in Asia [8, 32]. The wide spread of H9N2
40 virus have been greatly concerned not only in the poultry industry but also for public health [8, 38].
41 The hemagglutinin (HA) genes of Eurasian H9N2 viruses have been phylogenetically divided into
42 G1, Y280, and Korean sublineages [10]. H9N2 viruses do not substantially cause severe disease in
43 poultry, but co-infection with bacteria such as *Staphylococcus aureus*, *Haemophilus paragallinarum*,
44 or attenuated coronavirus vaccine exacerbates the disease [13, 22]. H9N2 viruses were also
45 isolated from domestic pigs in China [38] and Korea, and from humans with febrile respiratory
46 illness in Hong Kong in 1998, 1999, 2003, 2008, and 2009 [4-5, 23, 31]. It has therefore been
47 postulated that H9N2 virus has the potential to cause pandemic influenza in humans.

48 In the present study, as the preparedness for pandemic influenza, H9 virus strains from the
49 influenza virus library in our laboratory [19] were analyzed antigenically and phylogenetically to
50 select a strain suitable for a vaccine. A/duck/Hokkaido/49/1998 (H9N2) was selected and an
51 inactivated whole virus vaccine was prepared. The efficacy of the vaccine against challenge with
52 A/Hong Kong/1073/1999 (H9N2) was assessed in mice.

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56 **MATERIALS AND METHODS**

57 *Viruses:* A/duck/Hong Kong/Y280/1997 (H9N2), A/chicken/Hong Kong/G9/1997 (H9N2),
58 A/quail/Hong Kong/G1/1997 (H9N2), A/chicken/Hong Kong/FY20/1999 (H9N2), A/silkie

59 chicken/Hong Kong/SF43/1999 (H9N2), and A/quail/Hong Kong/A17/1999 (H9N2) were provided
60 by Dr. K. F. Shortridge (The University of Hong Kong, China). A/ostrich/South
61 Africa/9508103/1995 (H9N2) and A/chicken/Pakistan/2/1999 (H9N2) were provided by Dr. I. H.
62 Brown (Animal Health and Veterinary Laboratories Agency, Weybridge, U. K.). A/Hong
63 Kong/1073/1999 (H9N2) (HK/1073/99), which was isolated from a 4-year-old girl in Hong Kong in
64 1999 [23], was provided by Dr. A. J. Hay (MRC National Institute for Medical Research, U. K.).
65 H9N2 influenza virus strains isolated from birds and mammals, and A/duck/Hokkaido/49/1998
66 (H9N2) (Dk/Hok/49/98) [25] are listed in Table 1. The viruses were grown in 10-day-old
67 embryonated chicken eggs and infectious allantoic fluids were stored at -80°C until use.

68 *Phylogenetic analysis:* Viral RNAs were extracted from the allantoic fluids of chicken embryos
69 infected with viruses using TRIzol LS Reagent (Invitrogen, CA, U.S.A.) and reverse-transcribed
70 using the Uni12 primer [14] and M-MLV reverse transcriptase (Invitrogen). The cDNA was
71 amplified by using the Takara Ex Taq (Takara Bio, Inc., Shiga, Japan). The first cycle of the
72 amplification program consisted of a 5 min period at 94 °C and was followed by 30 cycles with the
73 following conditions; 98 °C for 10 sec, 55 °C for 30 sec, and 72 °C for 1 min. The last cycle was
74 done at 72 °C for 10 min. Polymerase chain reaction amplification of the viral genes was
75 performed using a PTC-200 thermal cycler (BIO-RAD, CA, U.S.A.). The primers used for HA
76 gene amplification were H9-101F (5'-GGCCACCAGTCAACAAACTC-3') [24] and H9-1341R
77 (5'-GTTTACATTCGCATCATGCTC-3'). Direct sequencing of the HA gene was performed using
78 a CEQ 2000XL autosequencer (Beckman Coulter, CA, U.S.A.). For phylogenetic analysis,
79 sequence data obtained for the genes together with those from public databases were analyzed using
80 the neighbor-joining method [34] using MEGA 5.0 software (http://www.megasoftware.net/).

81 *Antigenic analysis:* Antigenic characterization of H9N2 influenza viruses was done by
82 hemagglutination-inhibition (HI) test [35]. Hyperimmunized chicken antisera against seven H9N2

83 viruses were prepared [20]. Briefly, the sera were serially two-fold diluted with phosphate buffered
84 saline (PBS) in 96-well microplates. The diluted sera were mixed with 8 hemagglutinin units of
85 virus antigen and incubated at room temperature for 30 min. Chicken red blood cells (0.5%) were
86 added to the antigen-serum dilution mixtures and incubated at room temperature for 30 min. HI
87 titers were expressed as reciprocals of the highest serum dilutions that showed complete HI.

88 *Virus replication and pathogenicity in embryonated chicken eggs:* Viruses were inoculated into
89 10-day-old embryonated chicken eggs and incubated for 48 h at 35°C. HA titers and 50% egg
90 infectious dose (EID_{50}) were measured every 12 h post-inoculation. Pathogenicity of
91 Dk/Hok/49/98 against embryonated chicken eggs was evaluated by the mean death time as described
92 Abenes *et al.* [2].

93 *Vaccine preparation:* Dk/Hok/49/98 and HK/1073/99 were injected into the allantoic cavities of
94 10-day-old embryonated chicken eggs and propagated at 35°C for 48 h. The viruses in the allantoic
95 fluids (512 HA for Dk/Hok/49/98 and 1,024 HA for HK/1073/99) were purified by differential
96 centrifugation and sedimentation through a sucrose gradient [14]. The protein concentration was
97 measured using the BCA Protein Assay Reagent (Thermo Fisher Scientific K. K., MA, U.S.A.).
98 The purified virus was inactivated with 0.1% formalin at 4°C for 7 days. The HA content was
99 standardized as described [28]. Proteins of purified viruses were separated by sodium dodecyl
100 sulfate-polyacrylamide gel electrophoresis on a 15% gel (BIO-RAD) and stained with Coomassie
101 brilliant blue. The gel image was captured and analyzed by LumiVisionPRO (AISIN, Aichi, Japan),
102 and the ratio of HA protein to total protein was calculated. On the basis of this method,
103 concentration of HA protein was 14.7 μg in 50 μg of vaccine.

104 *Challenge with HK/1073/99 into mice vaccinated once or twice:* Inactivated Dk/Hok/49/98 or
105 HK/1073/99 vaccines were injected once intraperitoneally into 4-week-old female BALB/c mice
106 (Japan SLC, Inc., Shizuoka, Japan). PBS was injected into control mice. Three weeks later, 10