Table 2. Influenza virus strains analyzed in this study

Virus strain[a]	Subtype	Accession number	Virus strain	Subtype	Accession number
A/duck/Hokkaido/379/00	H4N6	AB478622	A/duck/Hokkaido/277/06	H6N2	AB478604
A/duck/Hokkaido/69/00	H5N3	AB300036	A/duck/Hokkaido/W162/06	H6N5	AB478618
A/duck/Hokkaido/18//00	H10N4	AB282876	A/duck/Hokkaido/W299/06	H9N2	AB478621
A/duck/Hokkaido/1169/01	H1N1	AB478607	A/duck/Hokkaido/W95/06	H10N8	AB569460
A/duck/Hokkaido/95/01	H2N2	AY422042	A/duck/Hokkaido/W73/07	H1N1	AB478614
A/duck/Hokkaido/17/01	H2N3	AY422040	A/duck/Hokkaido/W282/07	H4N6	AB478623
A/duck/Hokkaido/86/01	H2N3	AY422041	A/duck/Hokkaido/167/07	H5N3	AB378679
A/duck/Hokkaido/1005/01	H3N6	AB478606	A/duck/Hokkaido/201/07	H5N3	AB378687
A/duck/Hokkaido/56/01	H3N8	AB478611	A/duck/Hokkaido/69/07	H8N4	AB569464
A/duck/Hokkaido/1058/01	H4N5	AB569458	A/duck/Hokkaido/75/08	H3N6	AB569452
A/duck/Hokkaido/1019/01	H4N6	AB569457	A/duck/Hokkaido/W79/08	H4N6	AB569462
A/duck/Hokkaido/24/02	H11N9	AB478596	A/duck/Hokkaido/69/08	H4N6	AB569448
A/duck/Hokkaido/83/04	H1N1	AB478598	A/duck/Hokkaido/WZ21/08	H5N2	AB569454
A/duck/Hokkaido/18/04	H3N8	AB478595	A/duck/Hokkaido/W67/08	H6N1	AB569588
A/duck/Hokkaido/143/04	H4N2	AB569459	A/duck/Hokkaido/WZ76/08	H6N2	AB569453
A/duck/Hokkaido/W5/04	H4N6	AB569461	A/duck/Hokkaido/W112/08	H6N5	AB569466
A/duck/Hokkaido/193/04	H5N3	AB299377	A/duck/Hokkaido/W54/08	H6N8	AB569449
A/duck/Hokkaido/257/04	H6N1	AB478601	A/duck/Hokkaido/W76/08	H6N9	AB569465
A/duck/Hokkaido/W109/04	H6N2	AB478616	A/duck/Hokkaido/229/08	H7N7	AB569456
A/duck/Hokkaido/W12/04	H6N2	AB478609	A/duck/Hokkaido/238/08	H9N2	AB569467
A/duck/Hokkaido/W59/04	H8N4	AB478612	A/duck/Hokkaido/131/08	H10N7	AB569451
A/duck/Hokkaido/89/04	H10N5	AB478599	A/duck/Hokkaido/WZ16/08	H10N9	AB569463
A/duck/Hokkaido/W259/05	H2N5	AB478620	A/duck/Hokkaido/W45/08	H11N9	AB569455
A/duck/Hokkaido/12/05	H3N2	AB478594	A/ws/Hokkaido/OIE110/08	H12N2	AB569450
A/duck/Hokkaido/W70/05	H3N8	AB478613	A/duck/Hokkaido/WZ75/09	H5N2	AB569468
A/duck/Hokkaido/W268/05	H6N1	AB478603			
A/duck/Hokkaido/260/05	H8N4	AB478602			
A/duck/Hokkaido/279/06	H4N6	AB478605			
A/duck/Hokkaido/W206/06	H6N1	AB478619			

[a]Name of the virus with corresponding accession number of PB2 genes sequenced in this study.

(H6N2), A/duck/Hokkaido/W76/2008 (H6N9) and A/duck/Hokkaido/69/2008 (H4N6) characterized in this study were closely related to strains A/mallard/Korea/gH170/2007 (H7N7) and A/magpie/Korea/YJDI74/2007 (H7N7) isolated from domestic birds in Korea. Some viruses that fell in sublineage I were phylogenetically closely related to an isolate obtained from pintails in Alaska, virus strain A/northernpintail/Alaska/44204-108/06 (H3N1). Novel reassortant H5N1 HPAIV, A/chicken/Laos/P0130/2007 (H5N1) isolated from Laos[1] also belonged to this sublineage but was most closely related to a virus isolated in migratory birds in Korea, virus strain A/shorebird/Korea/S6/2006 (H1N2). The Eurasian sublineage II consisted of only one group (Fig 1). The H5N1

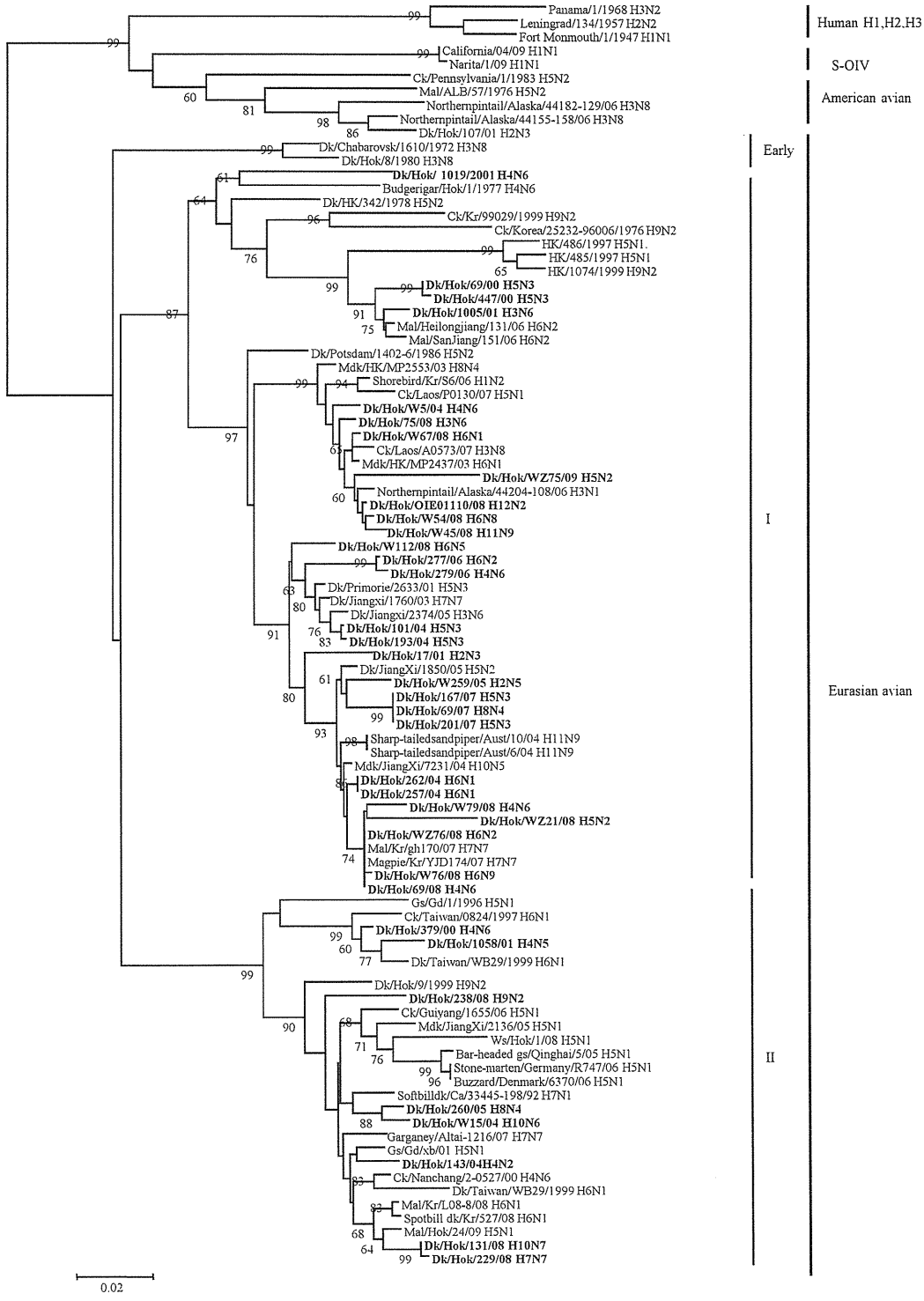

Fig. 1. Phylogenetic tree of influenza A virus PB2 genes. The phylogenetic tree was constructed using neighbour joining (NJ) method (1,000 replicates). For construction of this tree, 36 representative strains from a total of 54 that were sequenced. PB2 gene sequences each comprising 767 nucleotides (positions 1425–2192) were analyzed. This figure showing complete phylogram of avian influenza virus lineages with overall lineage of these isolates were of Eurasian avian and divided further into 2 distinct contemporary sublineages I and II. Bootstrap values below 60 are not shown. The strains sequenced in this study are indicated in bold.

HPAIV isolated from wild birds in China, Europe, and Japan belonged to this sublineage but none of the isolates tested in this study are closely related to these H5N1 HPAIVs.

Discussion

Rapid world wide spread of HPAIV to 62 countries in Eurasia and Africa with H5N1 viruses isolated from water birds found dead in Mongolia on the way back to their nesting lakes in Siberia in spring 2005, 2006, 2009 and 2010 raises concern that they may perpetuate in the northern nesting lakes in Siberia in summer. Since it was found that these H5N1 HPAIVs were genetically closely related to those influenza viruses isolated from birds in China, Iraq, Croatia, Nigeria, Korea and Japan, intensive surveillance of avian influenza in migratory water birds needs to be continued. In 2008–2009 avian influenza surveillance, we isolated 62 influenza viruses from fecal samples collected from migratory ducks that flew from their northern nesting lakes to Hokkaido, Japan in autumn. Influenza viruses of different subtypes have been isolated from these wild water birds. Twenty one combinations of the HA and NA subtypes of influenza viruses were detected. No H5N1 HPAIV was found during the surveillance period, indicating that the H5N1 HPAIV has not been perpetuated, at least dominantly in wild water birds that nest in northern territory in summer. The present findings are in agreement with previous study[13] showing that the H5N1 HPAIV has not persisted yet in wild water birds that nest in Siberia in summer.

The phylogenetic analyses in the present study revealed that none of the PB2 gene sequences of influenza viruses tested were closely related to HPAIV and none belonged to the American lineage. However, previous studies conducted in our laboratory found some internal protein genes (PB2, PA, and M) of influenza viruses isolated from migratory birds in Hokkaido which phylogenetically clustered with those of influenza viruses of the American lineage[12,13], indicating that interregional transmission of influenza virus genes do occur between the American and Eurasian gene pools among viruses obtained in Hokkaido. The grouping together of the PB2 gene of an influenza virus isolated from a pintail *(Anas acuta)* in Alaska with those of some viruses examined presently testifies to this phenomenon. The pintail (*Anas acuta*) species has been implicated in the inter-hemispheric transmission of influenza viruses between the American and Eurasian gene pools[8].

In conclusion, intensive surveillance of avian influenza conducted in Hokkaido in autumn in 2008–2009, has demonstrated that no HPAIVs were isolated from wild water birds flying from their nesting lakes in Siberia, indicating that the HPAIV has not yet persisted in their nesting lakes where they nest in summer. However, there is no guarantee that the absence of H5N1 HPAIV in wild water birds that come to Hokkaido is a permanent status. Therefore, the present study highlights the need for continued surveillance of avian influenza viruses in wild and domestic birds for the prevention and control of influenza.

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FULL PAPER

A vaccine prepared from a non-pathogenic H5N1 influenza virus strain from the influenza virus library conferred protective immunity to chickens against the challenge with antigenically drifted highly pathogenic avian influenza virus

Rozanah Asmah Abdul Samad[1)], Naoki Nomura[1)], Yoshimi Tsuda[1)], Rashid Manzoor[2)], Masahiro Kajihara[2)], Daisuke Tomabechi[2)], Takashi Sasaki[3)], Norihide Kokumai[3)], Toshiaki Ohgitani[3)], Masatoshi Okamatsu[1, 4)], Ayato Takada[2)], Yoshihiro Sakoda[1, 4)] and Hiroshi Kida[1, 4, 5, 6*)]

[1)]Laboratory of Microbiology, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan

[2)]Department of Global Epidemiology, Hokkaido University Research Center for Zoonosis Control, Sapporo 001-0020, Japan

[3)]Avian Biologics Department, Kyoto Biken Laboratories, Inc., Uji, Kyoto, 611-0041, Japan

[4)]OIE Reference Laboratory for Highly Pathogenic Avian Influenza, Sapporo 060-0818, Japan

[5)]Hokkaido University Research Center for Zoonosis Control, Sapporo 001-0020, Japan

[6)]SORST, Japan Science and Technology Agency (JST), 4-1-8 Honcho Kawaguchi, Saitama 332-0012, Japan

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Abstract

Inactivated influenza virus vaccine prepared from a non-pathogenic influenza virus strain A/duck/Hokkaido/Vac-1/2004 (H5N1) from the virus library conferred protective immunity to chickens against the challenge of antigenically drifted highly pathogenic avian influenza virus (HPAIV), A/whooper swan/Hokkaido/1/2008 (H5N1). The efficacy of the vaccine was comparable to that prepared from genetically modified HPAIV strain ΔRRRRK rg-A/whooper swan/Mongolia/3/2005 (H5N1), which is more antigenically related to the challenge virus strain, in chickens.

Keywords: *Antigenically drifted HPAIV, vaccine*

*Corresponding author: Hiroshi Kida, Laboratory of Microbiology, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan
Phone: +81-11-706-5207. Fax: +81-11-706-5273. E-mail: kida@vetmed.hokudai.ac.jp

Introduction

H5N1 highly pathogenic avian influenza virus (HPAIV) is causing panzootic outbreaks in poultry in Eurasia and Africa, posing serious concern for public health as well as live stock industry. The outbreaks of highly pathogenic avian influenza caused by H5N1 viruses that spread to 62 countries have taken toll of 300 million poultry (dead or been killed) and 15 countries with human fatalities[5]. In addition, the H5N1 HPAIVs had returned to migratory birds, spread to Eurasia and Africa[2,6]. Since each of the hemagglutinin (HA) genes of pandemic influenza viruses has been originated from avian influenza viruses[14], is now a potential pandemic threat. H5N1 viruses isolated from water birds found dead in Mongolia on the way back to their nesting lakes in Siberia in April to May in 2005, 2006, 2009 and 2010 were genetically closely related to H5N1 viruses isolated from birds in China, Iraq, Croatia, Nigeria, Korea and Japan[2,6,10]. Viruses similar to those have spread world-wide and it is therefore, a serious concern that these HPAIVs persists in Eurasia may perpetuate in the lakes where they nest in summer and that those birds may bring HPAIVs to the south in autumn.

Stamping-out and movement restriction are the standard measures for the control of highly pathogenic avian influenza (HPAI) in poultry and found to be successful in rapid eradication of the HPAIV infection[4]. Vaccination is a limited application as an optional tool when stamping-out is not effective enough to control the disease[1]. Vaccination may be an optional measure in cases where the disease spread widely. Many commercial vaccines have been prepared from viruses of the North American lineage. These vaccines may be less effective for the control of current HPAI outbreaks caused by the infection with viruses of the Eurasian lineage in Asia[5]. Inactivated influenza vaccines for the control of the circulating avian influenza particularly in Asia, therefore, should be prepared from an H5N1 virus strain belonging to the Eurasian lineage.

The OIE Reference Laboratory for HPAI at Hokkaido University has established the library of influenza viruses of all HA and neuraminidase (NA) subtypes and their genes[6]. The previous study[12] has demonstrated that the library of a panel of influenza virus strains isolated from natural hosts is useful for the preparedness for future pandemics. These influenza virus strains are stored in the library and have been used for the purpose of vaccine production and diagnosis.

Prolonged endemics of H5N1 virus in poultry has been known to generate antigenically and genetically diversified viruses in some countries[9]. Previous study[5,8], showed that avian influenza vaccine prepared from non-pathogenic avian influenza viruses from the library conferred protective immunity against the challenge virus of antigenically similar. Ideally, vaccine strains that are antigenically and genetically closely related to the circulating variant strain and induce immunity against antigenically drifted virus are preferable. In the present study, the efficacy of the vaccine prepared from a non-pathogenic influenza virus strain A/duck/Hokkaido/Vac-1/2004 (H5N1) from the virus library was comparable to that prepared from genetically modified HPAIV strain ΔRRRRK rg-Mon/05 (H5N1) by reverse genetics against the challenge with antigenically drifted HPAIV, A/whooper swan/Hokkaido/1/2008 (H5N1) in chickens.

Materials and Methods

Viruses: Influenza viruses, A/whooper swan/Hokkaido/1/2008 (H5N1) (Ws/Hok/08), A/duck/Hokkaido/Vac-1/2004 (H5N1) (Dk/Vac-1/04) and A/whooper swan/Mongolia/3/2005 (H5N1) (Ws/Mon/05) and mutant A/whooper swan/Mongolia/

3/2005 (H5N1) (ΔRRRRK rg-Mon/05), of clade 2.3.2, classical and clade 2.2 respectively were used. All viruses used in this study have been propagated and characterized antigenically and genetically as described[9]. All viruses were propagated in 10-day-old embryonated chicken eggs at 35°C for 30 to 48 hrs and stored at −80°C until use.

Dk/Vac-1/04 and ΔRRRRK rg-Mon/05 were used for vaccine preparation. A non-pathogenic avian influenza Dk/Vac-1/04 virus from the library, was generated as a reassortant virus between A/duck/Mongolia/54/2001 (H5N2) and A/duck/Mongolia/47/2001 (H7N1)[5]. Ws/Mon/05 virus isolated from a whooper swan (*Cygnus Cygnus*) found dead in Lake Khunt nuur, Mongolia[10] was genetically modified by reverse genetics with site-directed mutagenesis to generate a mutant ΔRRRRK rg-Mon/05 strain.

Meanwhile, Ws/Hok/08 (H5N1) virus that was isolated from whooper swan found dead at Notsuke Peninsular, in Hokkaido, Japan in May on their way back to their nesting lakes in northern territories[9] was used as the challenge virus.

Preparation of a genetically modified H5N1 HPAIV: Ws/Mon/05 was genetically modified by reverse genetics with site-directed mutagenesis as described[7]. Briefly, the amino acid RRRRK at the cleavage site of the HA were deleted and replaced with amino acid T by site-directed mutagenesis. The T mutation was introduced into the HA of Ws/Mon/05 cloned into the pHW2000 expression vector, using a Quick Change site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. The presence of the desired mutations was confirmed by sequencing the full length of the cloned HA genes. The mutant virus was designated ΔRRRRK rg-Mon/05 and confirmed to be apathogenic to chickens.

Intravenous pathogenicity (IVPI) of ΔRRRRK rg-Mon/05 in chickens: The intravenous pathogenicity index of ΔRRRRK rg-Mon/05 was carried out according to the OIE standard method[8]. Briefly, 1/10 dilutions of infectious allantoic fluids containing the viruses were intravenously inoculated into eight 6-week-old chickens. These chickens were housed for 10 days in self-contained isolator units (Tokiwa Kagaku Kikai Co., Ltd., Tokyo) at a BSL 3 facility at the Hokkaido University Research Center for Zoonosis Control, Japan. All animal experiments were conducted in accordance to guidelines of the Institutional Animal Care and Use Committee of Hokkaido University, Japan.

Vaccine preparation: A virus suspension of Dk/Vac-1/04 and ΔRRRRK rg-Mon/05 were inactivated by incubation with formalin at a 0.2% final concentration for 3 days at 4°C respectively. Virus inactivation was confirmed by inoculation into embryonated chicken eggs. The inactivated Dk/Vac-1/04 and ΔRRRRK rg-Mon/05 viruses suspension were diluted with phosphate-buffered saline (pH = 7.2) (PBS) to appropriate concentrations based on HA titers and adjuvanted as described[5,11].

Potency test of vaccine efficacy in chickens against antigenically drifted strains Ws/Hok/08: The potency of the vaccines was evaluated by challenging chickens inoculated with vaccines prepared from Dk/Vac-1/04 or ΔRRRRK rg-Mon/05 with antigenically drifted Ws/Hok/08. Thirty 4-week-old chickens were divided into three groups and the inactivated avian influenza virus Dk/Vac-1/04 or ΔRRRRK rg-Mon/05 vaccines were intramuscularly inoculated to ten chickens respectively as described[5,11]. PBS was inoculated in ten control chickens. Three weeks after vaccination, all chickens were challenged intranasally with a dose 10^3 50% chicken lethal dose (CLD_{50}) of Ws/Hok/08. Four chickens of each group were sacrificed on day 3 post-challenge and the remaining six chickens were observed

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.

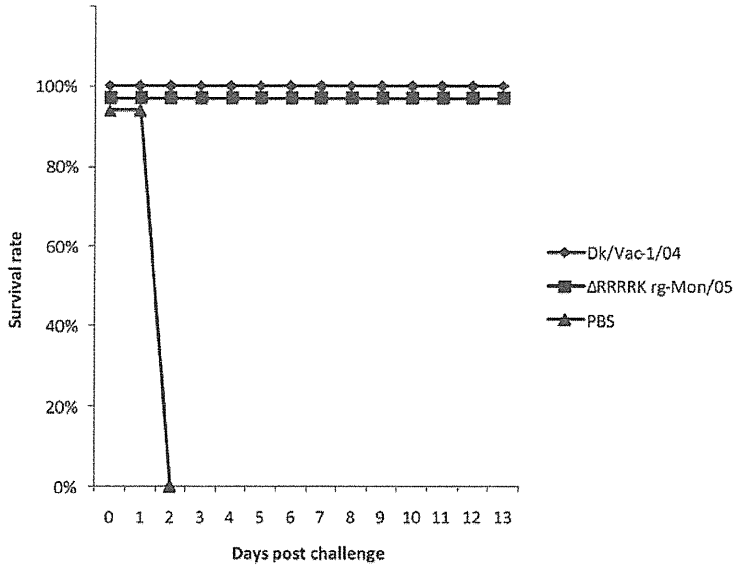

Fig. 1. Survival of chickens after challenge with Ws/Hok/08. Ten 4-week-old chickens in each group were vaccinated with Dk/Vac-1/04, and ΔRRRRK rg-Mon/05 respectively. Three weeks after vaccination, chickens were challenged with Ws/Hok/08 (H5N1) virus. Control chickens (black triangle) were dead within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

Yoshihiro Sakoda,[1]† Hiroshi Ito,[2,3]† Yuko Uchida,[4]† Masatoshi Okamatsu,[1] Naoki Yamamoto,[1] Kosuke Soda,[1,3] Naoki Nomura,[1] Saya Kuribayashi,[1] Shintaro Shichinohe,[1] Yuji Sunden,[5] Takashi Umemura,[5] Tatsufumi Usui,[3,6] Hiroichi Ozaki,[3,7] Tsuyoshi Yamaguchi,[3,6] Toshiyuki Murase,[3,7] Toshihiro Ito,[2,3] Takehiko Saito,[4] Ayato Takada[8] and Hiroshi Kida[1,8,9]

Correspondence
Hiroshi Kida
kida@vetmed.hokudai.ac.jp

[1]Laboratory of Microbiology, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan

[2]Laboratory of Veterinary Public Health, Faculty of Agriculture, Tottori University, Tottori 680-8553, Japan

[3]Avian Zoonosis Research Center, Faculty of Agriculture, Tottori University, Tottori 680-8553, Japan

[4]Research Team for Zoonotic Diseases, National Institute of Animal Health, Tsukuba 305-0856, Japan

[5]Laboratory of Comparative Pathology, Department of Veterinary Clinical Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan

[6]Laboratory of Veterinary Hygiene, Faculty of Agriculture, Tottori University, Tottori 680-8553, Japan

[7]Laboratory of Veterinary Microbiology, Faculty of Agriculture, Tottori University, Tottori 680-8553, Japan

[8]Research Center for Zoonosis Control, Hokkaido University, Sapporo 001-0020, Japan

[9]Japan Science and Technology Agency (JST), 4-1-8 Honcho, Kawaguchi 332-0012, Japan

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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†These authors contributed equally to this work.

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).

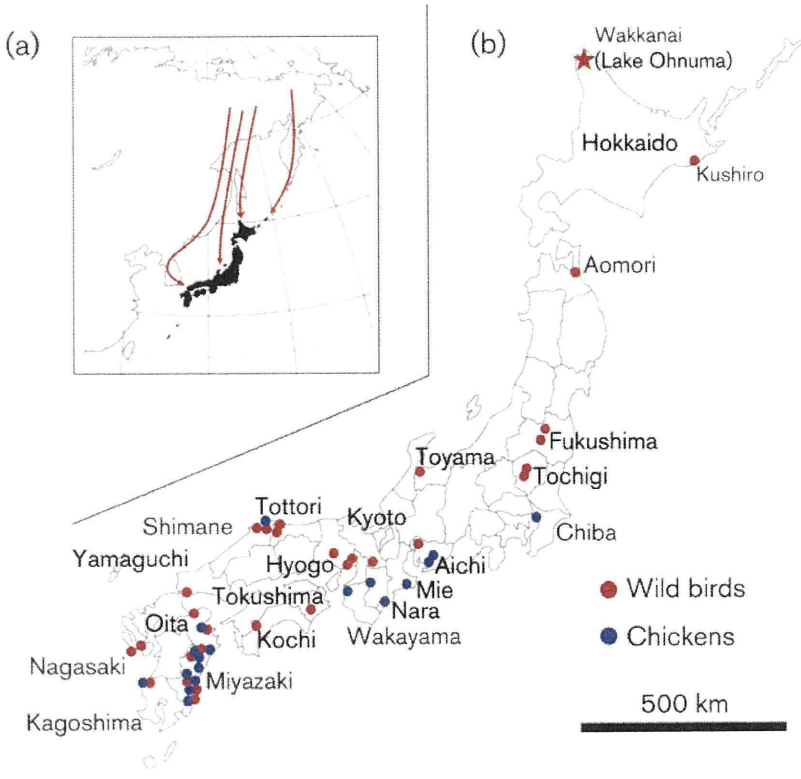

Fig. 1. H5N1 HPAIV infections in wild birds and chickens in the 2010–2011 winter season in Japan. (a) Geographical location of Japan in Asia and migration routes of wild water birds from Siberia in autumn (arrows). (b) On 14 October 2010 at Lake Ohnuma, Wakkanai, Hokkaido, Japan (red star), H5N1 HPAIVs 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).

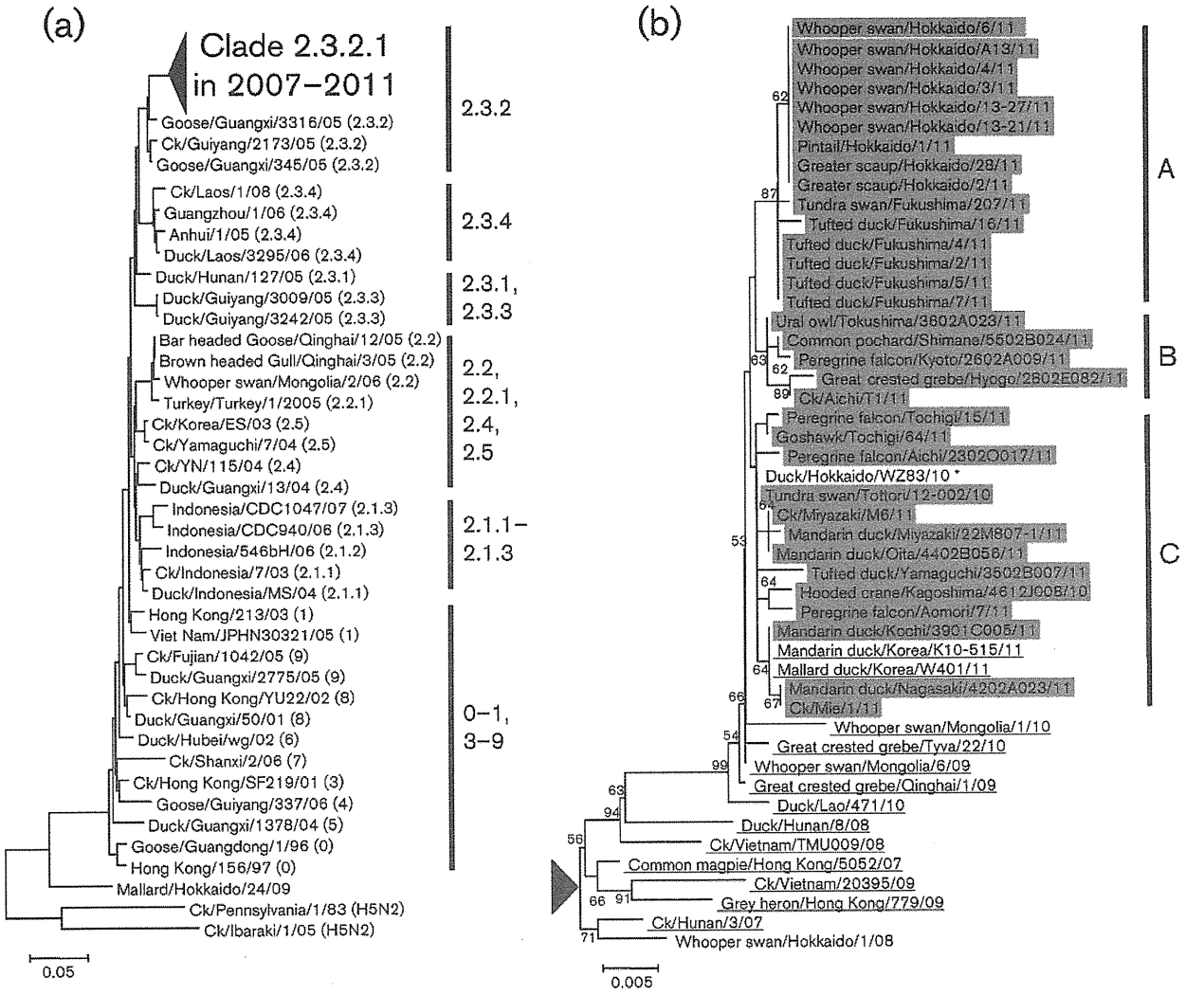

Fig. 2. Phylogenetic trees of HA genes of the isolates in the 2010–2011 winter season in Japan. (a) Phylogenetic tree of H5 avian influenza viruses. The unified nomenclature of the A/goose/Guangdong/1/1996 lineage of Eurasian HPAIVs was based on the homology of the HA gene and classified into ten distinct 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/Pennsylvania/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