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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).

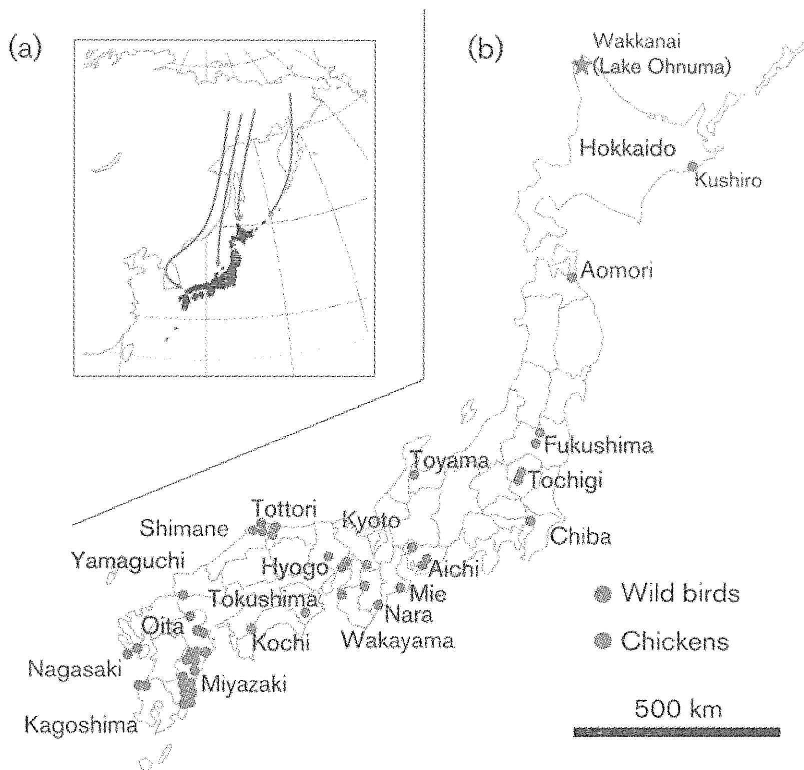

Fig. 1. H5N1 HPAIV infections in wild birds and chickens in the 2010–2011 winter season in Japan. (a) Geographical location of Japan in Asia and migration routes of wild water birds from Siberia in autumn (arrows). (b) On 14 October 2010 at Lake Ohnuma, Wakkanai, Hokkaido, Japan (red star), H5N1 HPAIVs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

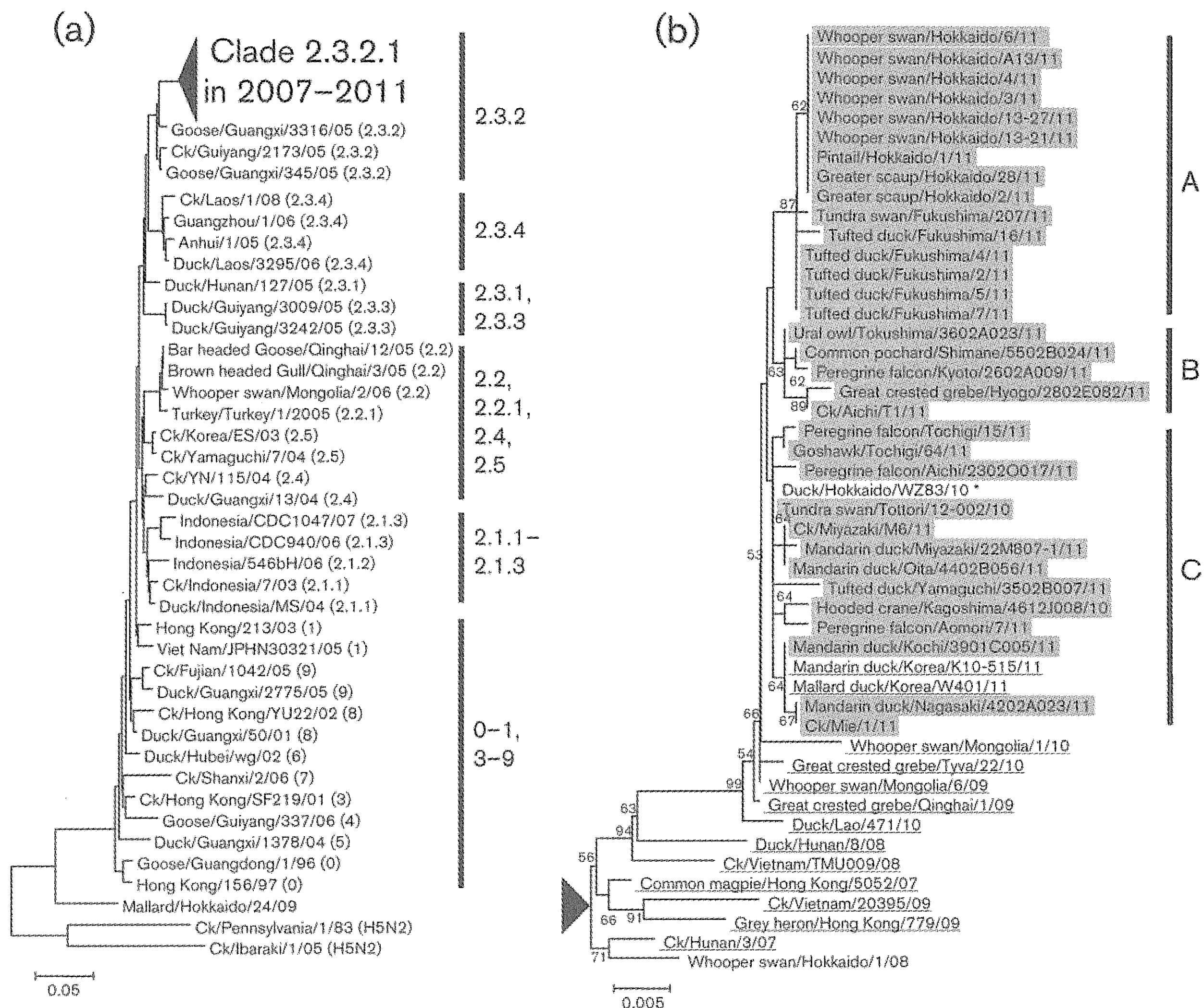

Fig. 2. Phylogenetic trees of HA genes of the isolates in the 2010–2011 winter season in Japan. (a) Phylogenetic tree of H5 avian influenza viruses. The unified nomenclature of the A/goose/Guangdong/1/1996 lineage of Eurasian HPAIVs was based on the homology of the HA gene and classified into ten distinct 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)	−	+	+	+	+	+	+	+	<u>1280</u>	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	<u>1280</u>
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

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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FULL PAPER *Virology*

An H9N2 Influenza Virus Vaccine Prepared from a Non-Pathogenic Isolate from a Migratory Duck Confers Protective Immunity in Mice against Challenge with an H9N2 Virus Isolated from a Girl in Hong Kong

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

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Avian influenza viruses of various subtypes are circulating in poultry worldwide [1, 18, 19, 21, 29, 38]. In particular, H9N2 influenza virus is prevailing poultry populations in Eurasian countries [9–11, 24]. Since H9N2 viruses were isolated from quails in Hong Kong in 1988, they have become prevalent in live bird markets and poultry farms in Asia [6, 32]. The wide spread of H9N2 virus have been greatly concerned not only in the poultry industry but also for public health [6, 38]. The hemagglutinin (HA) genes of Eurasian H9N2 viruses have been phylogenetically divided into G1, Y280, and Korean sublineages [10]. H9N2 viruses do not substantially cause severe disease in poultry, but co-infection with bacteria such as *Staphylococcus aureus*, *Haemophilus paragallinarum*, or attenuated coronavirus vaccine exacerbates the disease [13, 22]. H9N2 viruses were also isolated from domestic pigs in China [38] and Korea, and from humans with febrile respiratory illness in Hong Kong in 1998, 1999, 2003, 2008, and 2009 [4, 5, 23, 31]. It has therefore been postulated that H9N2 virus has the potential to cause pandemic influenza in humans.

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

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MATERIALS AND METHODS

Viruses: A/duck/Hong Kong/Y280/1997 (H9N2), A/chicken/Hong Kong/G9/1997 (H9N2), A/quail/Hong Kong/G1/1997 (H9N2), A/chicken/Hong Kong/FY20/1999 (H9N2), A/silkie chicken/Hong Kong/SF43/1999 (H9N2), and A/quail/Hong Kong/A17/1999 (H9N2) were provided by Dr. K. F. Shortridge (The University of Hong Kong, China). A/ostrich/South Africa/9508103/1995 (H9N2) and A/chicken/Pakistan/2/1999 (H9N2) were provided by Dr. I. H. Brown (Animal Health and Veterinary Laboratories Agency, Weybridge, U.K.). A/Hong Kong/1073/1999 (H9N2) (HK/1073/99), which was isolated from a 4-year-old girl in Hong Kong in 1999 [23], was provided by Dr. A. J. Hay (MRC National Institute for Medical Research, U.K.). H9N2 influenza virus strains isolated from birds and mammals, and A/duck/Hokkaido/49/1998 (H9N2) (Dk/Hok/49/98) [25] are listed in Table 1. The viruses were grown in 10-day-old embryonated chicken eggs and infectious allantoic fluids were stored at −80°C until use.

Phylogenetic analysis: Viral RNAs were extracted from the allantoic fluids of chicken embryos infected with viruses using TRIzol LS Reagent (Invitrogen, Carlsbad, CA, U.S.A.) and reverse-transcribed using the Uni12 primer [14] and M-MLV reverse transcriptase (Invitrogen). The cDNA was amplified by using the Takara Ex Taq (Takara Bio, Inc., Shiga, Japan). The first cycle of the amplifica-

Table 1. H9N2 viruses used in this study

Sublineage	Virus	HA gene[a)]
Y280	A/chicken/Hebei/1/1996	AF536693
	A/duck/Hong Kong/Y280/1997	AF156376
	A/chicken/Hong Kong/G9/1997	AF156373
	A/duck/Hong Kong/W213/1998	AB432938
	A/swine/Hong Kong/10/1998	AF222811
	A/chicken/Hong Kong/FY20/1999	AF222611
	A/silkie chicken/Hong Kong/SF43/1999	AF186268
Korean	A/ostrich/South Africa/9508103/1995	AF218102
	A/duck/Hokkaido/31/1997	AB125927
	A/duck/Hokkaido/49/1998	AB125928
	A/duck/Hokkaido/9/1999	AB125929
	A/duck/Hokkaido/26/1999	AB125930
	A/duck/Hokkaido/13/2000	AB125931
	A/duck/Hokkaido/HY57/2005	AB455035
	A/duck/Mongolia/564/2003	AB538969[b)]
	A/duck/Hokkaido/294/2006	AB538967[b)]
	A/duck/Hokkaido/W299/2006	AB538968[b)]
	A/duck/Hokkaido/238/2008	AB485600[b)]
G1	A/quail/Hong Kong/G1/1997	AF156378
	A/Hong Kong/1073/1999	AJ404626
	A/chicken/Pakistan/2/1999	AJ291392
	A/quail/Hong Kong/A17/1999	AF222606
North American	A/turkey/Wisconsin/1/1966	D90305

a) GenBank/EMBL/DDBJ Accession No. b) The HA gene sequence was submitted to the GenBank/EMBL/DDBJ databases in this study.

tion program consisted of a 5 min period at 94°C and was followed by 30 cycles with the following conditions; 98°C for 10 sec, 55°C for 30 sec, and 72°C for 1 min. The last cycle was done at 72°C for 10 min. Polymerase chain reaction amplification of the viral genes was performed using a PTC-200 thermal cycler (BIO-RAD, Hercules, CA, U.S.A.). The primers used for HA gene amplification were H9-101F (5'-GGCCACCAGTCAACAAACTC-3') [24] and H9-1341R (5'-GTTTACATTCGCATCATGCTC-3'). Direct sequencing of the HA gene was performed using a CEQ 2000XL autosequencer (Beckman Coulter, Fullerton, CA, U.S.A.). For phylogenetic analysis, sequence data obtained for the genes together with those from public databases were analyzed using the neighbor-joining method [33] using MEGA 5.0 software (http://www.megasoftware.net/).

Antigenic analysis: Antigenic characterization of H9N2 influenza viruses was done by hemagglutination-inhibition (HI) test [35]. Hyperimmunized chicken antisera against seven H9N2 viruses were prepared [20]. Briefly, the sera were serially two-fold diluted with phosphate buffered saline (PBS) in 96-well microplates. The diluted sera were mixed with 8 hemagglutinin units of virus antigen and incubated at room temperature for 30 min. Chicken red blood cells (0.5%) were added to the antigen-serum dilution mixtures and incubated at room temperature for 30 min. HI titers were expressed as reciprocals of the highest serum dilutions that showed complete HI.

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

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

Challenge with HK/1073/99 into mice vaccinated once or twice: Inactivated Dk/Hok/49/98 or HK/1073/99 vaccines were injected once intraperitoneally into 4-week-old female BALB/c mice (Japan SLC, Inc., Shizuoka, Japan). PBS was injected into control mice. Three weeks later, 10 mice in each group were challenged intranasally with 30 μl of $10^{6.5}$ EID_{50} of HK/1073/99 under anesthesia. Mixture of tiletamine hydrochloride (20 mg/kg) (United States Pharmacopeia, Rockville, MA, U.S.A.), zolazepam hydrochloride (20 mg/kg) (United States Pharmacopeia), and xylazine (20 mg/kg) (Bayer HelthCare, Osaka, Japan) was injected intraperitoneally into mice for anesthesia.

Inactivated Dk/Hok/49/98 vaccines were also injected twice intraperitoneally into 4-week-old female BALB/c mice. Two weeks later, the vaccine was again intraperitoneally injected into the mice. One week after the second vaccination, 10 mice in each group were challenged intranasally with 30 μl of $10^{6.5}$ EID_{50} of HK/1073/99 under anesthesia.

On day 3 post-infection, five mice in each group were sacrificed and the lungs were homogenized to make a 10% (w/v) suspension with minimal essential medium (Nissui, Tokyo, Japan) with antibiotics (penicillin G potassium, streptomycin sulfate, gentamicin sulfate, and nystatin) and 0.5% Bovine Serum Albumin Fraction V (Roche, Basel, Switzerland). The virus titers of the supernatants of the lung tissue homogenates were calculated in 10-day-old embryonated chicken eggs and expressed as the EID_{50}/g of tissue.

In neutralization (NT) tests, titers were determined as the reciprocal of that maximum antibody dilution that completely prevented cytopathic effect caused by 100 plaque forming units of virus using MDCK cells.

RESULTS

Phylogenetic analysis of the HA genes of H9N2 influenza viruses: The HA genes of 22 H9N2 viruses were sequenced and phylogenetically analyzed by the neighbor-joining method. All of the HA genes were classified into the Eurasian lineage, and further classified into the Korean (n=11), Y280 (n=7), and G1 (n=4) sublineages (Fig. 1). The H9 viruses of the Korean and Y280 sublineages were isolated from water birds, poultry, pigs, and humans in East Asian countries, and those of the G1 sublineage were isolated from poultry in west Asian countries (Fig. 1).

Antigenicity of the H9N2 influenza viruses: H9N2 influenza viruses were antigenically analyzed by HI test (Table 2). Antisera against H9N2 viruses of the Y280 sublineage reacted slightly with H9N2 viruses of the G1 and Korean sublineages. Antisera against H9N2 viruses of the G1 sublineage reacted more with H9N2 viruses of the Y280 sublineages than those of the Korean sublineage. On the other hand, antisera against H9N2 viruses of the Korean sublineage reacted with H9N2 viruses of all sublineages. This result suggested that the H9N2 vaccine strain should be selected from the viruses of the Korean sublineage.

Selection of H9N2 vaccine strain: To select an H9N2 vaccine strain, four H9N2 viruses, Dk/Hok/49/98, A/duck/Hokkaido/13/2000 (H9N2) (Dk/Hok/13/00), A/duck/Hokkaido/9/1999 (H9N2) (Dk/Hok/9/99), and A/duck/Hokkaido/26/1999 (H9N2) (Dk/Hok/26/99), were selected from 11 isolates of the Korean sublineage, and their replication and pathogenicity in embryonated chicken eggs were assessed. HA titers of Dk/Hok/49/98, Dk/Hok/13/00, Dk/Hok/9/99, and Dk/Hok/26/99 were 512, 512, 256, and 128, respectively. Virus titers were $10^{9.7}$, $10^{8.3}$, $10^{8.3}$, and $10^{7.3}$ EID_{50}/ml, respectively, indicating that Dk/Hok/49/98 replicated efficiently in 10-day-old embryonated chicken eggs. Pathogenicity of Dk/Hok/49/98 in the embryonated chicken eggs was determined by mean death time and that of Dk/Hok/49/98 was 91.8 hr, indicating that Dk/Hok/49/98 had low pathogenicity in chicken embryos. This virus was selected as a candidate H9N2 vaccine strain.

Protective efficacy of the test vaccine in mice against H9N2 virus challenge: To assess the efficacy of the vaccine against H9N2 virus infection, HK/1073/99 was intranasally inoculated into mice that had previously been vaccinated once with inactivated HK/1073/99 or Dk/Hok/49/98. Immunogenicity of the inactivated vaccine was assessed by NT test, and virus titers in the lungs were measured to assess protective immunity induced by the vaccine (Table 3). Serum antibodies were detected in mice injected with 50, 10, and 2 μg protein of HK/1073/99 vaccine. The virus titers in the lungs were $<10^{1.5}$–$10^{3.7}$ EID_{50}/g in mice injected with 50 or 10 μg protein of HK/1073/99 vaccine, and $10^{4.7}$–$10^{6.8}$ EID_{50}/g in the 2 and 0.4 μg vaccine groups, and in the PBS control group (Table 3). A reduction in body weight was observed in mice injected with 10, 2, and 0.4 μg protein, and in the control group from day 2 post-infection, reaching up to 10% body weight loss at days 3–4 post-infection, compared with in the mice that received 50 μg of protein (Fig. 2A).

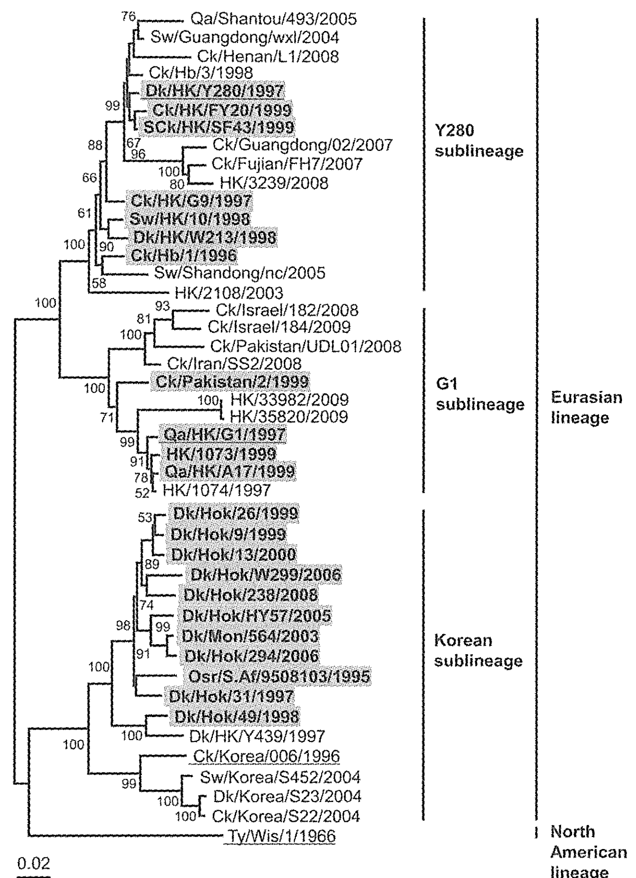

Fig. 1. Phylogenetic tree of the HA genes of H9N2 influenza viruses. Nucleotides 163–1,048 (886 bases) of the HA genes were used for the analysis. Horizontal distances are proportional to the minimum number of nucleotide differences required to join nodes and sequences. Numbers at the nodes indicate confidence levels in a bootstrap analysis with 1,000 replicates. Viruses stocked in our laboratory are highlighted in gray. Representative viruses in each sublineage are underlined. Abbreviations: Sw, swine; Ck, chicken; SCk, silky chicken; Dk, duck; Osr, ostrich; Qa, quail; Ty, turkey; HK, Hong Kong; Hb, Hebei; S.Af, South Africa; Hok, Hokkaido; Pak, Pakistan; and Wis, Wisconsin.

We also tested the efficacy of vaccination with Dk/Hok/49/98 on protection against subsequent intranasal infection with HK/1073/99. Serum antibodies were slightly detected in mice injected with Dk/Hok/49/98 vaccine containing 50 and 10 μg protein (Table 3). The virus titers in the lungs of mice injected with Dk/Hok/49/98 vaccine containing 50 and 10 μg protein were $10^{4.3}$–$10^{5.3}$ EID_{50}/g. In the mice injected with 2 and 0.4 μg protein, the virus titers in the lungs of mice were similar to those of non-vaccinated control mice (Table 3). Although, a reduction in body weight was observed in mice at all doses of the Dk/Hok/49/98 vaccine, slight significant difference was observed in mice injecteded with 50 μg protein, compared with in mice injected with PBS (Fig. 2B).

Table 2. The cross-reactivity of H9N2 viruses with antisera by HI test

Sublineage	Virus[b)]	Antisera[a)] Y280			Korean		G1	North American
		Ck/HK/G9/97	Dk/HK/Y280/97	Dk/HK/W213/98	Dk/Hok/49/98	Dk/Hok/13/00	Qa/HK/G1/97	Ty/Wis/1/66
Y280	Ck/Hb/1/96	10,240	10,240	2,560	2,560	2,560	2,560	40
	Ck/HK/G9/97	40,960	10,240	2,560	1,280	2,560	2,560	40
	Dk/HK/Y280/97	20,480	20,480	2,560	2,560	2,560	5,120	320
	Sw/HK/10/98	2,560	10,240	640	640	1,280	1,280	40
	Dk/HK/W213/98	40,960	20,480	2,560	1,280	2,560	2,560	80
	Ck/HK/FY20/99	10,240	10,240	2,560	2,560	2,560	5,120	160
Korean	Dk/Hok/31/97	640	640	640	1,280	2,560	160	640
	Dk/Hok/49/98	320	320	160	2,560	2,560	160	640
	Dk/Hok/9/99	640	640	160	2,560	2,560	80	320
	Dk/Hok/26/99	320	640	160	2,560	2,560	40	640
	Dk/Hok/13/00	640	640	160	1,280	2,560	80	640
	Dk/Mon/564/03	320	320	160	1,280	2,560	160	320
	Dk/Hok/HY57/05	640	320	320	1,280	2,560	80	320
	Dk/Hok/W299/06	640	320	640	1,280	2,560	80	640
	Dk/Hok/238/08	640	640	640	1,280	2,560	80	640
G1	Qa/HK/G1/97	640	1,280	320	1,280	1,280	5,120	320
	Ck/Pak/2/99	1,280	1,280	640	640	640	640	80
	HK/1073/99	1,280	320	160	1,280	80	1,280	320
North American	Ty/Wis/1/66	80	20	20	320	320	<20	640

a) Homologous reactions are underlined. b) This panel showed the representative strains of each sublineage.

Table 3. Neutralizing antibody titers before challenge and virus titers of the lungs after challenge in mice vaccinated once

Vaccine	Dose of vaccine	NT titer to HK/1073/99	NT titer to Dk/Hok/49/98	Virus titer[a)] ($\log EID_{50}$/g)
HK/1073/99	50 μg	320, 320, 160, 320, 640	ND	<1.5, <1.5, <1.5, <1.5, <1.5
	10 μg	80, 20, 80, 80, 40	ND	1.8, 3.7, 2.5, 2.3, 3.0
	2 μg	40, 20, 20, 20, 20	ND	4.7, 5.5, 5.5, 5.3, 6.0
	0.4 μg	<10, <10, <10, <10, <10	ND	5.7, 6.0, 5.0, 5.8, 5.5
	PBS	<10, <10, <10, <10, <10	ND	6.5, 6.2, 6.8, 6.5, 6.8
Dk/Hok/49/98	50 μg	40, 40, 40, 20, 40	160, 80, 80, 80, 80	4.3, 4.8, 4.5, 4.5, 4.8
	10 μg	<10, <10, <10, <10, <10	80, 80, 80, 80, 20	4.8, 5.0, 5.2, 4.7, 5.3
	2 μg	<10, <10, <10, <10, <10	<10, <10, <10, <10, <10	6.5, 5.8, 6.0, 6.3, 6.0
	0.4 μg	<10, <10, <10, <10, <10	<10, <10, <10, <10, <10	6.3, 5.8, 6.0, 6.5, 6.3
	PBS	<10, <10, <10, <10, <10	<10, <10, <10, <10, <10	6.2, 6.0, 6.5, 5.8, 6.5

Each of vaccine was injected intraperitoneally with 10 mice. Serum samples were collected 3 weeks after the vaccination. Mice were challenged with $10^{6.0}$ EID_{50} of HK/1073/99 intranasally. a) The lung samples were collected at 3 d.p.c. and virus titers were measured.

In the mice injected twice with Dk/Hok/49/98 vaccine on days 0 and 14, serum antibodies were detected in mice in the 50, 10, and 2 μg groups at one week after the second injection (Table 4). The virus titers in the lungs were $<10^{1.5}$–$10^{3.8}$ EID_{50}/g in mice injected with 50, 10, and 2 μg protein, and $10^{5.3}$–$10^{6.5}$ EID_{50}/g in the other vaccinated mice (Table 4). A reduction in body weight was observed in mice injected with 2 and 0.4 μg protein, and in control group, reaching up to 10% weight loss from days 4–6 post-infection, compared with in mice injected with 50 and 10 μg protein (Fig. 3). These results suggest that the repeat administration of the test vaccine confers immunity, and prevents body weight loss and decreases virus replication, after infection of mice with H9 influenza virus.

DISCUSSION

H9N2 viruses of each of the three sublineages, G1, Y280, and Korean, were recently isolated from wildbirds and poultry worldwide [3, 6, 27]. H9N2 viruses were also isolated from pigs and humans in China [4, 5, 37] and Korea, suggesting that these viruses have the potential to cause pandemic influenza in humans. H9N2 viruses isolated from pigs in China and Korea were classified into the Y280 and

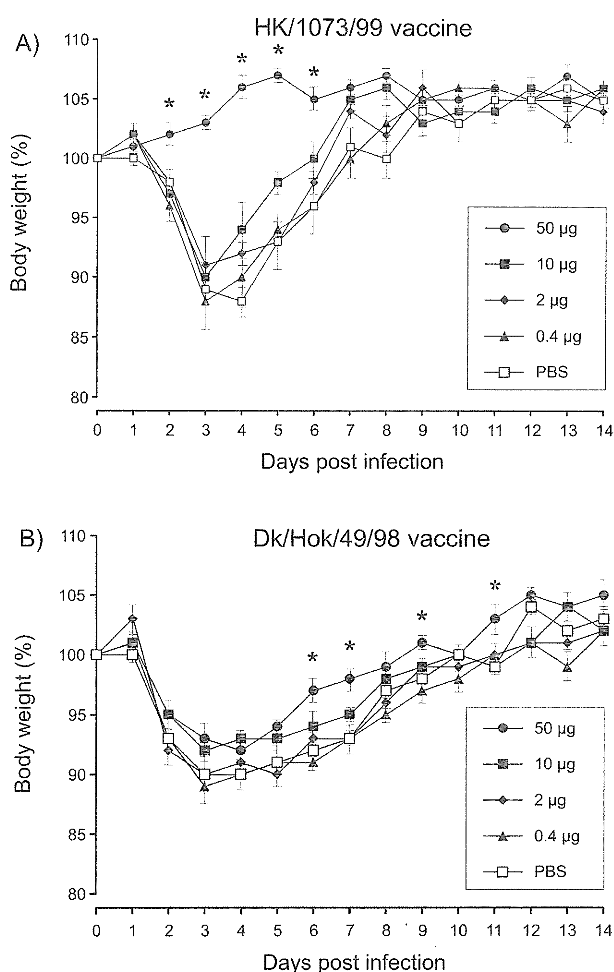

Fig. 2. Changes in body weight in mice vaccinated once following challenge with HK/1073/99. Five vaccinated mice of each group injected with HK/1073/99 vaccine (A), and with Dk/Hok/49/98 (B) were inoculated intranasally with HK/1073/99 and body weight was monitored for 14 days. Data are shown as mean body weight changes in each group with corresponding standard deviation (SD). Asterisks indicate that body weights were not significantly ($P<0.05$) decreased than PBS injected group.

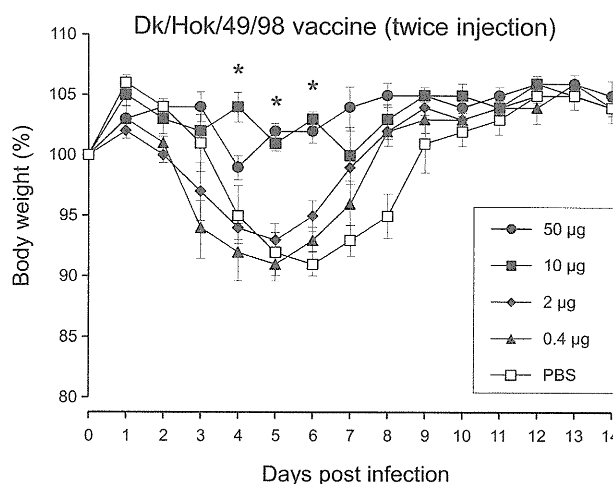

Fig. 3. Changes in body weight in mice vaccinated twice with Dk/Hok/49/98 vaccine following challenge with HK/1073/99. Five mice from each Dk/Hok/49/98 vaccine group were inoculated intranasally with HK/1073/99 and body weight was monitored for 14 days. Data are shown as mean body weight changes in each group with corresponding SD. Asterisks indicate that body weights were not significantly ($P<0.05$) decreased than PBS injected group.

Korean sublineages, while H9N2 viruses isolated from humans in China was classified into the G1 and Y280 sublineages [4, 5, 23, 31]. It is suggested that H9N2 viruses isolated from pigs and humans are antigenically distinct among viruses of the Korean, Y280, and G1 sublineages [5, 23, 31, 37]. Therefore, it is important that any H9N2 influenza virus vaccine to be used for pandemic influenza can broadly cross-react with antisera against all sublineage viruses. In the present study, Dk/Hok/49/98 was selected from the Korean sublineage, since antisera to the virus cross-reacted with all sublineages virus. Furthermore, Dk/Hok/49/98 replicated efficiently in embryonated chicken eggs and was non-pathogenic in chicken embryos. Recently, H9N2 viruses were isolated from pigs and humans in China [4, 5, 23, 31], it is necessary to analyze the antigenicity of these H9 isolates and evaluate the efficacy of test vaccine against

Table 4. Neutralizing antibody titers before challenge and virus titers of the lungs after challenge in mice vaccinated twice

Vaccine	Dose of vaccine	NT titer to		Virus titer[a)] ($logEID_{50}/g$)
		HK/1073/99	Dk/Hok/49/98	
Dk/Hok/49/98	50 μg	320, 320, 320, 160, 160	1,280, 640, 640, 320, 640	<1.5, <1.5, <1.5, <1.5, <1.5
	10 μg	40, <10, 20, 20, 40	320, 160, 320, 160, 160	2.0, 2.5, 2.0, 2.3, 2.5
	2 μg	<10, <10, <10, <10, <10	80, 160, 80, 80, 80	3.8, 3.5, 3.8, 3.5, 3.3
	0.4 μg	<10, <10, <10, <10, <10	<10, <10, <10, <10, <10	5.3, 5.8, 5.8, 6.0, 5.5
	PBS	<10, <10, <10, <10, <10	<10, <10, <10, <10, <10	5.8, 6.5, 6.0, 6.3, 5.8

Each of vaccine was injected intraperitoneally twice with 10 mice. Serum samples were collected 2 weeks after the second vaccination. Mice were challenged with $10^{6.0}$ EID_{50} of HK/1073/99 intranasally. a) The lung samples were collected at 3 d.p.c. and virus titers were measured.

them. Taken together, it is important to carry out surveillance of avian influenza consecutively and to analyze the isolates antigenically and phylogenetically.

In the present study, it was suggested that the test whole particle vaccine has the potency against challenge with H9N2 virus of different sublineage in mice. It was already reported that whole particle vaccine induced strong immune responses and H5N1 whole particle vaccine induced protective immunity against antigenically distinct challenge virus [12, 26]. Although the efficacy of the test vaccine observed slightly in mice vaccinated once due to the antigenic difference between Dk/Hok/49/98 and HK/1073/99, it was clear that the vaccine induced protective immunity in mice injected twice, indicating the usefulness for the preparedness of the pandemic.

The current cycle of seasonal influenza vaccine production requires detailed planning up to 6 months before vaccine manufacture [7]. In the case of influenza pandemic in 2009, it also took 5 months to have an H1N1 vaccine available [8, 36]. To prepare for the emergence of pandemic influenza in birds and mammals including humans, we have carried out global surveillance of avian influenza [15, 30, 34, 39]. Avian influenza viruses of 144 combinations of HA and NA subtypes have been stocked for use in vaccine and diagnosis. Since the viruses stocked in our influenza virus library were already assessed the pathogenicity and replication in embryonated chicken eggs, we can exclude those tests to select a vaccine strain and prepare a vaccine rapidly [16, 17, 19]. The present results indicate that the inactivated whole virus vaccine prepared from an influenza virus from the library could be used as an emergency vaccine during the early stage of a pandemic caused by H9N2 influenza virus infection.

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Characterization of Neutralizing Antibodies in Adults After Intranasal Vaccination With an Inactivated Influenza Vaccine

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The levels and properties of neutralizing antibodies in nasal wash and serum collected from five healthy adults were examined after intranasal administration of an A/Uruguay/716/2007 (H3N2) split vaccine (45 μg hemagglutinin (HA) per dose; five doses, with an interval of 3 weeks between each dose). Prior to the assays, nasal wash samples were concentrated so that the total amount of antibodies was equivalent to about 1/10 of that found in the natural nasal mucus. Vaccination induced virus-specific neutralizing antibody responses, which increased with the number of vaccine doses given. Neutralizing antibodies were produced more efficiently in the nasal passages than in the serum: A $\geq$4-fold increase in nasal neutralization titres was observed after the second vaccination in four out of five subjects, whereas a rise in serum neutralization titres was observed only after the fifth vaccination. Nasal and serum neutralizing antibodies were mainly found in the polymeric IgA and monomeric IgG fractions, respectively, after gel filtration. Taken together, these results suggest that intranasal administration of an inactivated split vaccine induces high levels of nasal neutralizing antibodies (primarily polymeric IgA) and low levels of serum neutralizing antibodies (primarily monomeric IgG). ***J. Med. Virol. 84:336–344, 2012.*** © 2011 Wiley Periodicals, Inc.

KEY WORDS: influenza; vaccine; neutralizing antibody

INTRODUCTION

To prevent influenza, protective immunity must be induced in advance by administration of a vaccine. Currently available inactivated vaccines, detergent disrupted split-viruses, or purified glycoproteins (surface antigen vaccines) are given via parenteral injection [Murphy and Webster, 1996]. Parenteral vaccination, that is, vaccination via the non-mucosal route, induces serum IgG antibodies, which are highly protective against homologous virus infection, but less effective against heterologous virus infection. Thus, intramuscular vaccination of seasonal influenza vaccine would be less effective in protecting against a heterologous virus epidemic.

A large number of studies show that the protective immunity induced by influenza virus infection is mainly mediated by secretory IgA (S-IgA) and IgG antibodies within the respiratory tract. S-IgA is carried to the mucus by transepithelial transport, while serum IgG is transported from the serum to the mucus by diffusion [Murphy and Clements, 1989; Brandtzag et al., 1994; Murphy, 1994; Asahi et al., 2002; Asahi-Ozaki et al., 2004]. S-IgA in the upper respiratory tract prevents viral infection, while IgG supports S-IgA-mediated protection by neutralizing newly-generated viruses [Ito et al., 2003; Renegar et al., 2004]. IgG is the main antibody involved in anti-viral protection in the lungs [Ramphal et al., 1979; Palladino et al., 1995; Renegar et al., 1998; Ito et al., 2003]. Also, polymeric S-IgA neutralizes viruses more effectively than monomeric IgA or IgG [Taylor

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and Dimmock, 1985; Renegar et al., 1998]. The polymeric nature of S-IgA also explains why S-IgA cross-reacts with variant influenza viruses to a greater extent than serum IgG [Tamura et al., 1990, 1991, 1992; Asahi-Ozaki et al., 2004]. Thus, intranasal administration of an inactivated influenza vaccine is advocated to elicit S-IgA and IgG responses and improve the protective efficacy of current vaccination procedures [Tamura and Kurata, 2004; Tamura et al., 2005, 2010].

Several clinical trials have examined the induction of both S-IgA and IgG following intranasal administration of inactivated influenza vaccines, either with or without adjuvant [Kuno-Sakai et al., 1994; Hashigucci et al., 1996; Muszkat et al., 2000; Greenbaum et al., 2002; Durrer et al., 2003; Treanor et al., 2006; Atmar et al., 2007]. The antibody responses after intranasal administration of inactivated influenza vaccines were assessed by measuring hemagglutination inhibition (HI) titres in the serum, and anti-hemagglutinin (HA) IgA and IgG titres in nasal wash samples. They did not measure the titre of neutralizing antibodies, which is considered to be a better criterion for functional protective antibodies. Neutralization titres can directly inhibit the complex process involved in virus replication, which include virus attachment and entry to the host cells, and release of newly-synthesized virus from the infected cells in tissue culture. In addition, a previous study found that HI titres were lower, or higher, than the corresponding neutralization titres, depending on a strain of influenza A or B virus used for the assay [Okuno et al., 1990], whereas other studies show that anti-H5 HI antibodies fail to detect H5N1 viruses [Lu et al., 1982; Rowe et al., 1999]. Thus, neutralizing antibody responses following intranasal administration of an inactivated influenza vaccine remain to be fully characterized.

Therefore, the aim of the present study was to examine the levels and properties of neutralizing-antibodies in nasal wash and serum samples from healthy adults after intranasal administration of an inactivated vaccine (five doses, with an interval of 3 weeks between each dose). The inactivated vaccine used in this study was a concentrated split-virus vaccine (containing 45 μg HA per dose), prepared from the A/Uruguay/716/2007 (H3N2) strain. A concentrated split-virus vaccine was chosen because the vaccine has already been shown to induce mucosal antibody responses after intranasal vaccination [Kuno-Sakai et al., 1994]. To ensure that neutralization titres specific for the A/Uruguay/716/2007 virus were assayed at equivalent levels in both serum and nasal wash samples, the neutralization titres were measured using concentrated nasal wash samples (1 mg/ml total protein) that contained approximately 1/10 of the IgA found in undiluted mucus [Kurono and Mogi, 1987]. The properties of the neutralizing IgA and IgG antibodies induced by intranasal vaccination were then examined, and their relative levels and molecular size were determined.

MATERIALS AND METHODS

Subjects

Five healthy male subjects (P1, P2, P3, P4, and P5) were enrolled in the study (aged 22, 32, 42, 42, and 68 years, respectively, at the time of the first vaccination). All participants had already acquired some degree of immunity to H1N1 and H3N2 influenza A virus subtypes after previous exposure to these viruses and/or as a result of previous vaccinations. Each subject provided informed consent and the study protocol and other relevant documentation were reviewed and approved by the Ethics Committee of the National Institute of Infectious Diseases (Tokyo, Japan).

Virus and Vaccine

The A/Uruguay/716/2007 (A/Uruguay; H3N2) influenza virus strain was propagated in the allantoic cavity of 10-day-old embryonated hen's eggs and purified from the allantoic fluid. The $TCID_{50}$ (50% infectious dose in tissue culture) of the virus was estimated as described previously [Tobita et al., 1975; Kadowaki et al., 2000]. In brief, 10-fold serial dilutions of the allantoic fluid containing the virus were inoculated into Madin-Darby canine kidney (MDCK) cells (ATCC No. CCL-34) cells in 96-well culture plates and incubated for 4 days at 37°C in a 5% CO_2 humidified atmosphere. The cytopathic effects in the virus-containing wells were monitored under a microscope and the $TCID_{50}$ was calculated using the Reed–Muench method. The split product virus vaccine was supplied by the Research Foundation for Microbial Disease of Osaka University (BIKEN, Kanonji, Japan). The vaccine was prepared from purified viruses, which were sedimented through a linear sucrose gradient according to the manufacturer's protocol. The viruses were then treated with ether and formalin according to the manufacturer's protocol, which was based on the method of Davenport et al. [1964]. The concentrated split vaccine containing 45 μg HA was the product of a process used to prepare a trivalent vaccine comprising A/H1N1, A/H3N2, and B type vaccines, each containing 15 μg HA.

Vaccinations

All participants were immunized intranasally with a threefold concentrated split H3N2 virus vaccine (A/Uruguay, containing 45 μg HA). Each received five doses, with an interval of 3 weeks between each dose. Intranasal vaccination was performed by spraying 0.25 ml of the split vaccine into each nostril (0.5 ml total) using an atomizer (Keytron, Ichikawa, Japan). The mean droplet diameter was 56.5 μm, ranging in size between 10 μm and 90 μm.

Nasal Wash and Serum Samples

About 100 ml of nasal wash was collected from each participant in polypropylene tubes by washing the

nasal cavity several times using a nose irrigation device (Hananoa; Kobayashi Pharmaceutical, Osaka, Japan) filled with saline solution according to the manufacturer's instructions. Pieces of dental cotton (Dental Cotton Roll; B.S.A. Sakurai, Nagoya, Japan) were then immersed in the collected nasal washes. Dental cotton pieces (containing a combined absorbed volume of about 25 ml of nasal wash) were then placed into a filter insert (Oxi Fil filter insert; TOHO, Tokyo, Japan) with bottoms drilled to create several pores, and placed in 50 ml polypropylene centrifuge tubes. Clean nasal wash was separated from mucopolysaccharides and other debris by centrifugation at 2,200*g* for 5 min at room temperature. This procedure was repeated for the entire 100 ml nasal wash sample from each participant. The pooled, clean nasal wash was then concentrated to a final volume of approximately 0.5 ml using Vivaspin centrifugal concentrators (Vivaspin 20, MWCO = 30,000; Sartorius Stedim Biotech, Aubagne, France). The concentrated nasal wash was stored at −80°C before use.

Quantitation of IgA, IgG and IgM Antibodies and Other Proteins

The levels of human IgA, IgG, and IgM antibodies in the nasal wash and serum samples were estimated using human IgA, IgG, or IgM ELISA kits (Bethyl Laboratories, Montgomery, USA). The level of human serum albumin in the nasal wash samples was estimated using a Human Albumin ELISA kit (Bethyl Laboratories). The protein concentration in the samples was measured using either a BCA Protein Assay Kit, or a Micro BCA Protein Assay Kit (Thermo Fisher Scientific, Yokohama, Japan) according to the manufacturer's instructions.

Neutralization Assays

The level of serum antibodies against the vaccine viruses was examined using micro-neutralization assays as previously described [Belshe et al., 2000; Kadowaki et al., 2000] with minor modifications. In brief, serum samples were treated with a receptor-destroying enzyme (RDE(II); Denka Seiken, Tokyo, Japan) overnight at 37°C and heat-inactivated for 30 min at 56°C before use. The first dilution tested in the assays was 1:10. The concentrated nasal wash samples [1 mg/ml total protein, corresponding to about 1/10 of the total IgA found in nasal mucus (2.20 mg/ml)] [Kurono and Mogi, 1987] were also treated with RDE(II) and heat-inactivated before use. The first dilution tested in the nasal wash assays was 1:20. Twofold serial dilutions of the serum samples were mixed with an equal volume (50 μl) of diluent containing influenza virus equivalent to 100 $TCID_{50}$. Each mixture was added to the wells of a 96-well plate containing a monolayer of MDCK cells. Four control wells were included on each plate and contained either virus or diluent alone. The plates were then incubated for 4 days at 37°C in a 5% CO_2-humidified atmosphere. The monolayer in each well was observed for the presence or absence of cytopathic effects, fixed with 10% formalin for more than 5 min at room temperature, and stained with Naphthol blue black. After the plates were washed and dried, the stained cells were solubilized with 0.1 M NaOH and the absorbance (A) was measured at 630 nm. The average $A_{630\ nm}$ value was determined from quadruplicate virus-infected wells (A_{virus}) and cell culture-only controls (A_{cell}). All values above 50% of the specific signal, calculated using the formula: $X = (1/2) \times (A_{cell} - A_{virus}) + A_{virus}$, were considered positive for neutralization. The titres recorded were the reciprocal of the highest dilution, where A_{630} was $>X$.

Hemagglutination Inhibition

The antibody responses to the vaccine viruses were examined in serum and nasal washes using HI antibody assays incorporating a microtiter method as described elsewhere [Hierholzer et al., 1969]. All samples were pre-treated with RDE(II) at 37°C for 18 hr, subsequently inactivated at 56°C for 30 min, and mixed with packed red blood cells to remove any nonspecific inhibitors. The starting material for the assays was a 1:10 dilution for the serum samples and a 1:40 dilution for the nasal wash samples.

Fractionation of Nasal and Serum Samples

The concentrated nasal wash samples (100 μl, 6 mg/ml) and diluted serum samples (10-fold dilution, 100 μl, about 6 mg/ml) were fractionated on a Superose 6 10/300 GL gel filtration column using an FPLC-AKTA chromatography system (GE Healthcare, Little Chalfont, UK). The concentrated nasal wash sample was treated with 1 μg/ml of lysozyme (Sigma-Aldrich, St. Louis, MO) for 1 hr at 37°C to decrease the viscosity and then centrifuged using Vivaspin to remove the lysozyme prior to gel filtration. Fractions (each 500 μl) were collected in PBS at a flow rate of 0.1 ml/min; little or no change in the fractionation pattern of the antibodies in the concentrated nasal wash samples was observed following lysozyme treatment. Molecular weight marker proteins (Kit for Molecular Weights 29,000–700,000 Da; Sigma-Aldrich) were eluted under the same conditions to determine the size of each fraction.

RESULTS

Measurement of Neutralization and HI Titres in Concentrated Nasal Wash Samples

The total protein level and the levels of IgA, IgG, and IgM and human serum albumin in 100 ml of unconcentrated nasal wash and in approximately 0.5 ml of concentrated nasal wash are shown in Table I. About 70% of the total nasal wash proteins were lost during the concentration process. Also, a fraction of the higher molecular weight (MW) proteins and lower MW proteins (less than 30 kDa) was lost by