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Potency of an inactivated influenza vaccine prepared from a non-pathogenic H5N1 virus against a challenge with antigenically drifted highly pathogenic avian influenza viruses in chickens

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ABSTRACT

Antigenic variants of H5N1 highly pathogenic avian influenza virus (HPAIV) have selected and are prevailing in poultry populations in Asia. In the present study, the potency of inactivated influenza vaccine prepared from a non-pathogenic H5N1 avian influenza virus, A/duck/Hokkaido/Vac-3/2007 (H5N1), was assessed by challenging with H5N1 HPAIV variants, A/muscovy duck/Vietnam/OIE-559/2011 (H5N1), A/whooper swan/Hokkaido/4/2011 (H5N1), and A/peregrine falcon/Hong Kong/810/2009 (H5N1) belonging to clades 1, 2.3.2.1, and 2.3.4, respectively. All chickens immunized with the Vac-3 vaccine survived without showing any clinical signs after intranasal challenge either with A/whooper swan/Hokkaido/4/2011 (H5N1) or A/muscovy duck/Vietnam/OIE-559/2011 (H5N1). After challenge with A/peregrine falcon/Hong Kong/810/2009 (H5N1), 10 out of 12 vaccinated chickens survived and the other 2 died on 4 or 7 post-challenge days. The Vac-3 vaccine of 2.4-fold antigen concentration conferred complete protective immunity in chickens against challenge with A/peregrine falcon/Hong Kong/810/2009 (H5N1).

1. Introduction

Avian influenza caused by 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). In recent intensive surveillance studies in Asia, especially in China, genetically different viruses of clades 2.3.2, 2.3.4, and 7 were characterized as dominant isolates from poultry and wild birds (Kou et al., 2009; Smith et al., 2009; Jiang et al., 2010). In the updated grouping of H5 HPAIVs, it was reported that the clades of H5N1 viruses were divided into one or more newly defined second-, third-, and/or fourth-order clades, e.g. recent H5N1 isolates that had been categorized into clade 2.3.2 were defined as

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clade 2.3.2.1 (WHO/OIE/FAO H5N1 Evolution Working Group, 2012). In Japan, H5N1 HPAIVs belonging to clade 2.3.2.1 were isolated from dead whooper swans in 2008 (Uchida et al., 2008; Okamatsu et al., 2010), fecal samples of ducks that flew from Siberia in October 2010 (Kajihara et al., 2011), and from wild birds and domestic poultry in 2011 (Sakoda et al., 2012). Antigenicity of H5N1 HPAIVs belonging to clades 2.3.2.1 and 2.3.4 was distinct from that of other HPAIVs and non-pathogenic avian influenza viruses (Okamatsu et al., 2010; Smith et al., 2009), suggesting that antigenic variants of H5N1 HPAIV have been selected during circulation in poultry populations.

A reassortant influenza virus, A/duck/Hokkaido/Vac-1/2004 (H5N1) (Dk/Vac-1/04), was generated using two non-pathogenic avian influenza viruses, A/duck/Mongolia/54/2001 (H5N3) and A/duck/Mongolia/47/2001 (H7N1). Both viruses were isolated from fecal samples of migratory ducks (Soda et al., 2008b). Vac-1 vaccine prepared from Dk/Vac-1/04 conferred immunity to suppress the manifestation of clinical signs and the amount of virus shed in chickens after challenge with H5N1 HPAIVs belonging to clades 2.2 and 2.5 (Isoda et al., 2008). Vac-1 vaccine induced a high level of HI antibody response in chickens, lasting as long as 138 weeks after vaccination (Sasaki et al., 2009b). Vac-1 vaccine confers protective immunity against antigenically drifted H5N1 HPAIV, A/whooper swan/Hokkaido/1/2008 (H5N1) (Ws/Hok/08), belonging to clade 2.3.2.1 in chickens (Okamatsu et al., 2010).

In the present study, we prepared a vaccine from A/duck/Hokkaido/Vac-3/2007 (H5N1) (Dk/Vac-3/07), which is antigenically closely related with Dk/Vac-1/04, and growth potential in embryonated chicken eggs was higher than that of Dk/Vac-1/04 (Soda et al., 2008b). The potency of the Vac-3 vaccine was assessed by challenge with recently prevailing antigenic variant HPAIVs.

2. Materials and methods

2.1. Viruses

Dk/Vac-3/07 was generated in our laboratory as a reassortant influenza virus between A/duck/Hokkaido/101/2004 (H5N3) and A/duck/Hokkaido/262/2004 (H6N1), isolated from fecal samples of migratory ducks (Soda et al., 2008b). The following HPAIV isolates were used: A/muscovy duck/Vietnam/OIE-559/2011 (H5N1) (Mdk/VN/11), was isolated from an apparently healthy muscovy duck in Viet Nam in 2011, A/whooper swan/Hokkaido/4/2011 (H5N1) (Ws/Hok/11), isolated from a dead whooper swan found on the waterside of lake Ohnuma in Hokkaido, Japan (Sakoda et al., 2012), and A/peregrine falcon/Hong Kong/810/2009 (H5N1) (Pf/HK/09) was kindly provided by Dr. Luk S. M. Geraldine, Tai Lung Veterinary Laboratory (Hong Kong SAR, China). All viruses used in the present study were propagated in 10-day-old embryonated chicken eggs at 35 °C for 30–48 h and infectious allantoic fluids were stored at −80 °C until use.

The complete nucleotide sequence of Dk/Vac-1/04 and Dk/Vac-3/07 have been registered in GenBank/EMBL/DDBJ (Accession numbers: AB259709-AB259716 and AB355926-AB355933, respectively) (Soda et al., 2008b). It is also revealed that the all genes of Dk/Vac-1/04 were closely related to Dk/Vac-3/07 (98% similarity in HA gene, 97% similarity in NA gene, and more than 99% similarity in the other genes).

2.2. Generation of recombinant viruses

In addition to Dk/Vac-3/07, vaccine was prepared from attenuated Pf/HK/09. Viral RNA was extracted from the allantoic fluid of embryonated chicken eggs infected with Pf/HK/09 using a commercial kit (TRIzol LS Reagent, Invitrogen, Carlsbad, CA, USA) and reverse transcribed with the Uni12 primer (Desselberger et al., 1980) and M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). PCR-based amplification of the full-length HA and NA gene segments was performed using universal primer sets (Hoffmann et al., 2001). The PCR products were inserted into the vector pHW2000 (Hoffmann et al., 2000) using an In-Fusion HD Cloning Kit (Takara Bio Inc., Otsu, Shiga, Japan). To generate a mutant virus with polybasic amino acid residue RRRK deletions at the HA cleavage site, amin acid mutation residue T (codon ACA) were substituted into the HA cleavage site of the Pf/HK/09 strains using a Quick Change II site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). Attenuated Pf/HK/09, Pf/mut (H5N1), was generated by reverse genetics methods according to Hoffmann et al. (2000). Pf/mut (H5N1) possesses the gene encoding the mutant HA of Pf/HK/09, in which polybasic amino acid at the cleavage site was deleted, NA of Pf/HK/09, and the backbone of Dk/Vac-1/04 internal genes. To confirm attenuation of Pf/mut (H5N1), the IVPI test was carried out according to the OIE (World Organization for Animal Health) manual (OIE, 2008).

2.3. Vaccine preparation

Vac-3 vaccine and Pf/mut vaccine were prepared from Dk/Vac-3/07 or Pf/mut (H5N1), respectively. Dk/Vac-3/07 or Pf/mut (H5N1) was inoculated into the allantoic cavity of 10-day-old embryonated chicken eggs and the eggs were then incubated at 35 °C for 48 h. After the allantoic fluid was harvested, formalin was added to a final concentration of 0.2%, and the mixture was incubated at 4 °C for 7 days to inactivate the viruses. Virus inactivation was confirmed by inoculation of the formalin-treated samples into embryonated chicken eggs. The inactivated Dk/Vac-3/07 and Pf/mut (H5N1) virus suspensions were concentrated by ultrafiltration using the Vivaflow 200 (Sartorius AG, Goettingen, Germany), then diluted with phosphate-buffered saline (PBS, pH7.2) to give the required hemagglutinin titer concentration. The inactivated viruses, light liquid paraffin, sorbitan monooleate, and polysorbate 80 were mixed in a volume ratio of 9:36:4:1 and then agitated to obtain emulsion. The Vac-3 vaccine of 2.4-fold antigen concentration was also prepared and designated as Vac-3 conc. vaccine. Vac-3 vaccine contains inactivated virus of 756 HA per dose and was prepared from the dilution of infectious allantoic fluid 1:1 with PBS, 378 HA per dose of Pf/mut vaccine at 1:1, and 1843 HA per dose of Vac-3 conc. vaccine at 2.4:1, respectively.

2.4. Antigenic analysis of the viruses

Polyclonal antisera were prepared from chickens immunized with inactivated Dk/Vac-3/07, A/chicken/Yamaguchi/7/2004 (H5N1) (Ck/Yamaguchi/04), A/whooper swan/Mongolia/3/2005 (H5N1) (Ws/Mon/05), Mdk/VN/11, Ws/Hok/11, or Pf/HK/09. Each virus inactivated with formalin was inoculated once or twice into the lower thigh muscle of chickens. Two weeks after the final immunization, serum was obtained from each vaccinated chicken and used for a hemagglutination-inhibition (HI) test to assess antigenic relationships among H5 influenza viruses. HI test was performed according to Isoda et al. (2008). The differences within 4-fold HI titers were determined as antigenically related, whereas over 4-fold determined as antigenically different.

The antigenic specificities of H5 viruses, Mdk/VN/11, Ws/Hok/11, and Pf/HK/09, were assessed by the fluorescent antibody method using monoclonal antibodies (MAb) to the HA according to the method of Soda et al. (2008a). MDCK cells infected with each of the H5 influenza virus were fixed with 100% acetone 8 h post-inoculation. Reactivity patterns of the H5 viruses with MAbs were investigated with FITC-conjugated goat anti-mouse IgG (ICN Biomedicals Inc., Irvine, CA, USA) using a fluorescent microscope (Axiovert 200; Carl Zeiss, Oberkochen, Germany).

2.5. Potency test of vaccines in chickens

One hundred and ten chickens (White leghorn) were hatched and raised in our laboratory. Half a milliliter of Vac-3 vaccine was injected into the lower thigh muscle of 54 four-week-old chickens. Three weeks later, 18 vaccinated and 4 non-vaccinated seven-week-old chickens in 3 groups were challenged intranasally with 100 50% chicken lethal dose (CLD_{50}) of Mdk/VN/11, Ws/Hok/11, or Pf/HK/09. One hundred times CLD_{50} of Mdk/VN/11, Ws/Hok/11, and Pf/HK/09 was $10^{6.3}$, $10^{5.7}$, and $10^{5.5}$ 50% egg infectious dose (EID_{50}), respectively. Twelve out of 18 vaccinated chickens in each group were monitored for their clinical signs for 2 weeks, and 6 chickens in each group were sacrificed 3 days post-challenge (d.p.c.).

Pf/mut and Vac-3 conc. vaccines were injected into the lower thigh muscle of 2 groups of 18 four-week-old chickens. Three weeks later, 18 vaccinated and 4 non-vaccinated chickens in the 2 groups were challenged intranasally with 100 CLD_{50} of Pf/HK/09. Twelve out of 18 vaccinated chickens in each group were monitored for their clinical signs for 2 weeks, and 6 chickens in each group were sacrificed 3 d.p.c.

When chickens died or were sacrificed, tracheal and cloacal swabs were collected and soaked in minimum essential medium (MEM), and their tissues (trachea, lungs, kidneys, and colon) were collected aseptically. To make 10% suspensions with MEM, the collected tissue samples were homogenized using a Multi-Beads Shocker (Yasui Kikai, Osaka, Japan). Serial 10-fold dilutions of the suspentions with PBS were inoculated into 10-day-old embryonated chicken eggs and incubated at 35 °C for 48 h. EID_{50} of viruses was determined by the method of Reed and Muench (1938) and expressed as EID_{50} per milliliter of swab or gram of tissue, respectively. Sera were collected from all of the 90 vaccinated and 20 non-vaccinated chickens just before challenge and examined for the presence of antibodies against the vaccine strains and challenge virus strains by the HI tests. Challenge studies were carried out in self-contained isolator units (Tokiwa kagaku, Tokyo, Japan) at a BSL3 biosafety facility at the Graduate School of Veterinary Medicine, Hokkaido University, Japan. Animal experiments were authorized by the Institutional Animal Care and Use Committee of Hokkaido University (approval numbers: 09-0119 and 10-0007) and all experiments were performed according to the guidelines of this committee.

3. Results

3.1. Antigenic analysis

H5N1 HPAIVs used as the vaccine strain and challenge virus in the present study were antigenically analyzed by the HI tests (Table 1). Dk/Vac-3/07 is antigenically closely related to Ck/Yamaguchi/04 and Ws/Mon/05, but is different from Ws/Hok/11 and Pf/HK/09. The recent H5N1 HPAIV isolates belonging to clades 1.1, 2.3.2.1, and 2.3.4 were antigenically different.

H5N1 HPAIVs used as the vaccine strain and challenge virus in the present study were antigenically analyzed using a panel of MAbs recognizing six different epitopes on the HA of A/duck/Pennsylvania/10218/84 (H5N2). Each of the MAbs bound to the low pathogenic avian influenza

Table 1
Antigenic analysis of H5N1 subtype avian influenza viruses using polyclonal antibodies.[a]

Virus	Clade[b]	Antiserum to					
		Vac-3	Yama/04	Mon/05	VN/11	Hok/11	HK/09
A/duck/Hokkaido/Vac-3/2007	–	128	16	32	4	8	<4
A/chicken/Yamaguchi/7/2004	2.5	128	128	128	32	32	<4
A/whooper swan/Mongolia/3/2005	2.2	128	256	512	128	128	64
A/muscovy duck/Vietnam/OIE-559/2011	1.1	256	64	16	256	16	16
A/whooper swan/Hokkaido/4/2011	2.3.2.1	32	16	64	16	128	<4
A/peregrine falcon/Hong Kong/810/2009	2.3.4	16	8	8	16	16	128

Vac-3, A/duck/Hokkaido/Vac-3/2007; Yama/04, A/chicken/Yamaguchi/7/2004; Mon/05, A/whooper swan/Mongolia/3/2005; VN/11, A/muscovy duck/OIE-559/2011; Hok/11, A/whooper swan/Hokkaido/4/2011; HK/09, A/peregrine falcon/Hong Kong/810/2009.

[a] Homologous titers are underlined.

[b] "–" indicate lineages not belonging to clade 0–9.

Table 2
Antigenic analysis of H5 influenza viruses using monoclonal antibodies.

Virus[a]	Clade[b]	Monoclonal antibodies[c]						
		I (88)	II (145)	III (157)	IV (168)		V (169)	VI (205)
		D101/1	A310/39	64/1	B9/5	B220/1	B59/5	25/2
LPAIV								
A/duck/Pennsilvania10218/1984 (H5N2)	–	+	+	+	+	+	+	+
A/duck/Hokkaido/Vac-3/2007 (H5N1)	–	+	+	+	+	+	+	+
HPAIV								
A/Vietnam/1194/2004 (H5N1)	1	+	+	+	+	+	–	+
A/chicken/Yamaguchi/7/2004 (H5N1)	2.5	–	+	+	+	+	–	+
A/whooper swan/Mongolia/3/2005 (H5N1)	2.2	+	+	+	+	+	–	+
A/muscovy duck/Vietnam/OIE-559/2011 (H5N1)	**1.1**	**+**	–	–	–	–	–	**+**
A/whooper swan/Hokkaido/4/2011 (H5N1)	**2.3.2.1**	**+**	–	–	–	–	–	–
A/peregrine falcon/Hong Kong/810/2009 (H5N1)	**2.3.4**	–	–	–	–	–	–	–

[a] Viruses indicated in bold were used in the challenge study.
[b] "–" indicate lineages not belonging to clade 0–9.
[c] Reactivity of monoclonal antibodies against the HA of A/duck/Pennsylvania/10218/1984 (H5N2) to the representative H5 viruses was compared using fluorescent antibody methods. Location of amino acid substitutions in antigenic variants selected in the presence of respective monoclonal antibodies is indicated in parentheses.

viruses (LPAIVs) and HPAIVs isolated before 2005, and few MAbs bound to the antigens of Mdk/VN/11, Ws/Hok/11, and Pf/HK/09. It was demonstrated that the epitopes recognized by these MAbs were conserved in LPAIVs and HPAIVs isolated before 2005, but not in recently prevailing HPAIVs (Table 2).

3.2. Efficacy of the Vac-3 vaccine in chickens

Fifty-four vaccinated chickens and 12 non-vaccinated chickens were challenged intranasally with each of the HPAIVs, Mdk/VN/11, Ws/Hok/11, and Pf/HK/09. The serum HI titers of the vaccinated chickens varied with each of the challenge viruses. The survival rates of the chickens challenged with each of the three HPAIVs are shown in Fig. 1. All vaccinated chickens survived without showing any disease signs after challenge either with Mdk/VN/11 or Ws/Hok/11, whereas two vaccinated chickens died after challenge with Pf/HK/09. All non-vaccinated chickens challenged with any of the HPAIVs died within 2 to 4 d.p.c. (Fig. 1A–C).

To evaluate the potential of Vac-3 vaccine to induce immunity for the prevention of virus shedding, we tried to recover the virus from swabs and tissues of the vaccinated and non-vaccinated chickens after challenge with each of HPAIV, Mdk/VN/11, Ws/Hok/11, and Pf/HK/09 (Table 3). Infectivity titers of the recovered viruses from vaccinated chickens were lower than those of non-vaccinated chickens after challenge with Mdk/VN/11 or Pf/HK/09 3 d.p.c. Infectious viruses were recovered from tracheal swabs and the organs of vaccinated chickens 3 d.p.c. with Ws/Hok/11, although the titers of viruses recovered from these birds were lower than those from non-vaccinated chickens.

3.3. Efficacy of the Vac-3 conc. vaccine against Pf/HK/09 in chickens

In order to enhance the efficacy of Vac-3 vaccine, the antigen concentration of Vac-3 vaccine was increased ×2.4 and designated as Vac-3 conc. vaccine. The Vac-3 conc. vaccine was assessed for efficacy against a challenge with Pf/HK/09. Pf/mut vaccine prepared from Pf/mut (H5N1) (IVPI = 0.00) was also assessed for its potency as the homologous control. Thirty-six chickens immunized either with Vac-3 conc. or Pf/mut vaccine and 8 non-vaccinated chickens were challenged intranasally with Pf/HK/09.

Table 3
Virus recovery from chickens vaccinated with Vac-3 vaccine challenged with H5N1 HPAIVs.

Challenge virus	Vaccination	Sampling d.p.c.[a]	No. of chickens	HI titer[b]		Virus recovery					
						No. of chickens from which each virus was recovered [GM value of the virus titer (log 10)]					
				Dk/Vac-3/07	Challenge virus	Swab (log EID50/ml)		Tissue (log EID50/g)			
						Tracheal	Cloacal	Trachea	Lungs	Colon	Kidneys
Mdk/VN/11	Vaccinated	3	6	256	8–64	1 (≤0.7)	0	0	0	0	0
	Non-vaccinated	2†	4	<4	<4	4 (5.5)	4 (5.3)	4 (8.6)	4 (9.3)	4 (8.5)	4 (9.4)
Ws/Hok/11	Vaccinated	3	6	256–512	16–64	2 (≤1.0, ≤1.3)	0	3 (4.3)	4 (5.1)	5 (4.4)	4 (4.0)
	Non-vaccinated	3–4†	4	<4	<4	4 (4.2)	4 (3.2)	4 (6.4)	4 (8.2)	4 (6.9)	4 (7.9)
Pf/HK/09	Vaccinated	3	6	64–512	<4–16	2 (≤0.7, ≤1.3)	0	0	1 (3.5)	0	0
	Non-vaccinated	2†	4	<4	<4	4 (4.9)	4 (5.4)	4 (7.6)	4 (9.1)	4 (8.3)	4 (8.8)

[a] Swab and tissue samples were collected on the following days from sacrificed (no mark) or dead (†) chickens.
[b] The range of HI titers using either Dk/Vac-3/07 or each challenge virus before challenge is indicated.

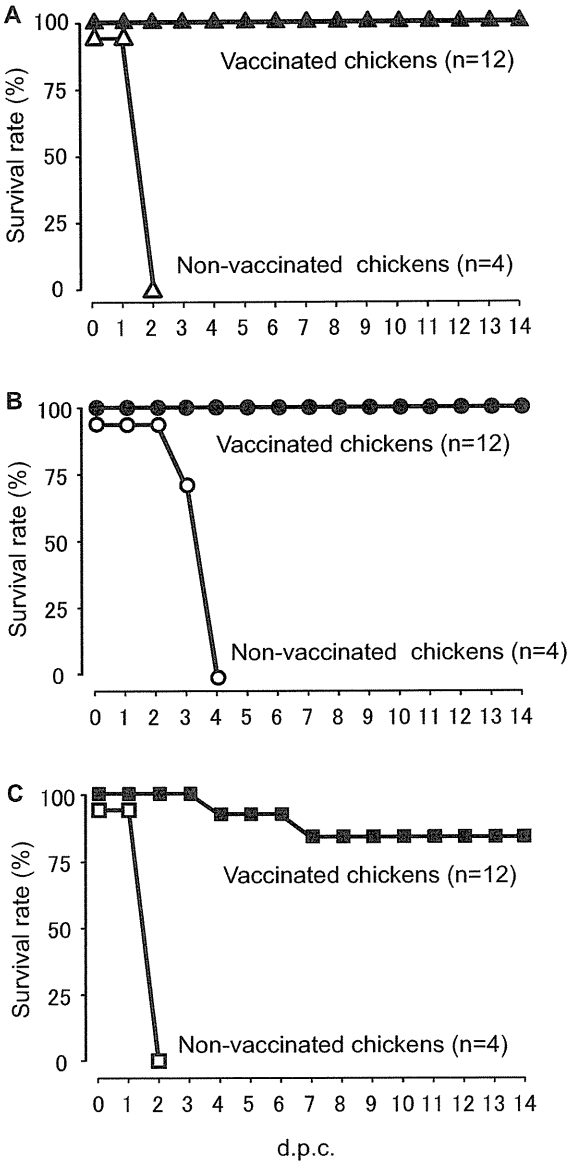

Fig. 1. Survival rates of chickens vaccinated with Vac-3 vaccine after challenge with H5N1 HPAIVs. Twelve four-week-old chickens from each group were immunized intramuscularly with 0.5 ml of Vac-3 vaccine. Three weeks after vaccination, the vaccinated chickens were challenged with 100 CLD_{50} of Mdk/VN/11(A), Ws/Hok/11(B), and Pf/HK/09 (C), respectively.

HI titer to Pf/HK/09 of the sera of the chickens immunized with Vac-3 conc. vaccine was 4-16 HI, which is similar to those of the chickens immunized with Vac-3 vaccine (Table 4). The survival rates of the chickens challenged with Pf/HK/09 are shown in Fig. 2. All vaccinated chickens survived without showing any disease signs after the challenge with Pf/HK/09 (Fig. 2A and B). All non-vaccinated chickens challenged with Pf/HK/09 died within 3 d.p.c. Viruses were not recovered from swabs or organs of any of the chickens immunized with Pf/mut vaccine after the challenge with Pf/HK/09 (Table 4). Virus was scarcely recovered from the tracheal swab of chicken immunized with Vac-3 conc. vaccine and the viral titer was lower than in non-vaccinated chickens after challenge with Pf/HK/09.

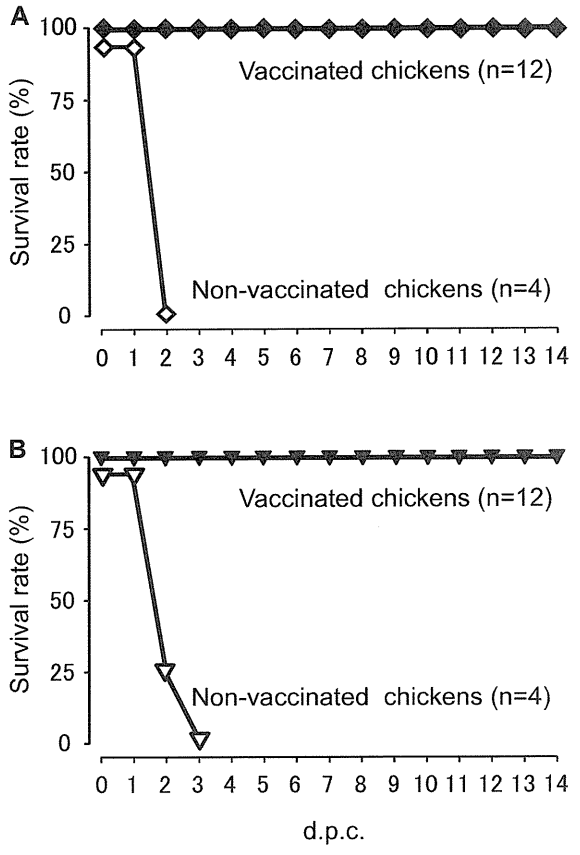

Fig. 2. Survival rates of chickens vaccinated with Pf/mut vaccine (A) and Vac-3 conc. vaccine (B) after challenge with Pf/HK/09. Twelve four-week-old chickens from each group were immunized intramuscularly with 0.5 ml of Pf/mut vaccine or Vac-3 conc. vaccine. Three weeks after vaccination, these vaccinated chickens were challenged with 100 CLD_{50} of Pf/HK/09.

4. Discussion

Antigenic variants of H5N1 HPAIVs have been selected in poultry under immunological selection pressure (Cattoli et al., 2011; Grund et al., 2011). In the present study, it was demonstrated that H5N1 HPAIVs prevailing recently in Asia were antigenically different from non-pathogenic avian influenza virus and H5N1 HPAIVs isolated before 2005 (Table 1). We previously demonstrated that an inactivated avian influenza vaccine prepared from Dk/Vac-1/04 conferred protective immunity and reduced the amount of virus shedding when chicken was challenged with Ck/Yamaguchi/04, Ws/Mon/05, and Ws/Hok/08 (Isoda et al., 2008; Okamatsu et al., 2010). In the present study, we prepared an inactivated influenza vaccine from Dk/Vac-3/07, which is a reassortant generated between non-pathogenic avian influenza viruses isolated from wild water birds. It is assumed that Vac-3 vaccine has similar potency with Vac-1 vaccine against recent H5N1 HPAIVs since Dk/Vac-3/07 is antigenically similar to Dk/Vac-1/04. However, the growth potential of Dk/Vac-3/07 is better than that of Dk/Vac-1/04. It is possible to generated concentrated Vac-3 vaccine using Dk/Vac-3/07. The

Table 4
Virus recovery from chickens challenged with Pf/HK/09.

Vaccination	Sampling d.p.c.[a]	No. of chickens	HI titer[b]		Virus recovery					
					No. of chickens from which each virus was recovered [GM value of the virus titer (log 10)]					
			Dk/Vac-3/07	Pf/HK/09	Swab (log EID50/ml)		Tissue (log EID50/g)			
					Tracheal	Cloacal	Trachea	Lungs	Colon	Kidneys
Pf/mut	3	6	16–32	128–512	0	0	0	0	0	0
Vac-3 conc.	3	6	256–1024	4–16	1 (1.7)	0	0	0	0	0
Control	2–3†	8	<4	<4	8 (5.1)	8 (5.3)	8 (7.8)	8 (9.0)	8 (8.3)	8 (8.7)

[a] Swab and tissue samples were collected on the following days from sacrificed (no mark) or dead (†) chickens.
[b] The range of HI titers using either Dk/Vac-3/07 or Pf/HK/09 before challenge is indicated.

potency of Vac-3 vaccine was assessed by challenging with antigenically drifted H5N1 HPAIVs isolated in 2009 and 2011. Vac-3 vaccine conferred protective immunity to suppress the manifestation of clinical signs and virus shedding in chickens challenged with antigenically drifted H5N1 HPAIVs belonging to clades 1.1, 2.3.2.1, and 2.3.4. In order to clarify why the efficacy of Vac-3 vaccine was not sufficient to protect all vaccinated chickens from the challenge with Pf/HK/09, we prepared Pf/mut vaccine, which was antigenically homologous with Pf/HK/09 (data not shown). All chickens immunized with Pf/mut vaccine survived for 14 days without showing any clinical signs and viruses were not detected from the swabs and tissues of the chickens. These results correspond to the findings that Pf/HK/09 is antigenically different from Dk/Vac-3/07 compared with Mdk/VN/11 and Ws/Hok/11. To improve the efficacy of Vac-3 vaccine, antigen concentration was increased for Vac-3 conc. vaccine preparation. HI antibody responses of vaccinated chickens correlated with the antigen concentration in H5N1 (Sasaki et al., 2009a) or H7N7 (Maas et al., 2009) influenza virus vaccine. Inactivated whole particle vaccine confers protective immunity against a challenge with viruses antigenically drifted from the vaccine strain to chickens by increasing the antigen concentration (Hwang et al., 2011). Vac-3 conc. vaccine conferred protective immunity to all vaccinated chickens after the challenge with Pf/HK/09. The vaccine with increased antigen concentration induced sufficient immunity to protect from infection with variant H5N1 HPAIV in chickens.

In the present study, it was demonstrated that the vaccine prepared from non-pathogenic avian influenza virus conferred protective immunity against the challenge with antigenically drifted H5N1 HPAIVs, indicating that Vac-3 vaccine induces sufficient immunity in chickens. The results of the antigenic analysis indicate broad antigenic diversity among H5N1 HPAIVs prevailing recently in Asia (Table 1). The vaccine prepared from recent H5N1 HPAIVs may not be completely effective against HPAIVs belonging to different clades. Since the misuse of vaccines lead to the silent spread of antigenically drifted viruses, it is recommended that avian influenza vaccine should be applied very carefully in addition to the stamping-out policy. There is an urgent need to eradicate H5N1 HPAIV from Asia by stamping-out without misusing vaccines (Table 4).

5. Conclusion

All chickens immunized with the Vac-3 vaccine survived without showing any clinical signs after intranasal challenge either with A/whooper swan/Hokkaido/4/2011 (H5N1) or A/muscovy duck/Vietnam/OIE-559/2011 (H5N1). The Vac-3 conc. vaccine of 2.4-fold antigen concentration conferred complete protective immunity in chickens against challenge with A/peregrine falcon/Hong Kong/810/2009 (H5N1).

Conflict of interest statement

The authors declare that they have no conflict interests.

Acknowledgements

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Potency of a vaccine prepared from A/swine/Hokkaido/2/1981 (H1N1) against A/Narita/1/2009 (H1N1) pandemic influenza virus strain

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Abstract

Background: The pandemic 2009 (H1N1) influenza virus has spread throughout the world and is now causing seasonal influenza. To prepare for the emergence of pandemic influenza, we have established a library of virus strains isolated from birds, pigs, and humans in global surveillance studies.

Methods: Inactivated whole virus particle (WV) and ether-split (ES) vaccines were prepared from an influenza virus strain, A/swine/Hokkaido/2/1981 (H1N1), from the library and from A/Narita/1/2009 (H1N1) pandemic strain. Each of the vaccines was injected subcutaneously into mice and their potencies were evaluated by challenge with A/Narita/1/2009 (H1N1) virus strain in mice.

Results: A/swine/Hokkaido/2/81 (H1N1), which was isolated from the lung of a diseased piglet, was selected on the basis of their antigenicity and growth capacity in embryonated chicken eggs. Two injections of the WV vaccine induced an immune response in mice, decreasing the impact of disease caused by the challenge with A/Narita/1/2009 (H1N1), as did the vaccine prepared from the homologous strain.

Conclusion: The WV vaccine prepared from an influenza virus in the library is useful as an emergency vaccine in the early phase of pandemic influenza.

Keywords: Influenza A (H1N1)pdm, Vaccine, Swine influenza virus

Background

A pandemic influenza caused by swine-origin H1N1 virus appeared in Mexico in 2009 and spread throughout the world [1-3]. The pandemic virus isolates were antigenically similar to classical swine influenza viruses and distinct from H1N1 virus strains circulating in humans since 1977 [2,4]. A pandemic 2009 (H1N1) vaccine was produced and evaluated in clinical trials [5]. The production of a large amount of egg-produced pandemic 2009 (H1N1) vaccine was, however, limited due to its poor yield in chicken embryos [6], leading to a delay in the efficient control of the pandemic.

It was revealed that the H3 HA gene of A/Hong Kong/68 (H3N2) strain originated from that of isolates from migratory ducks and that pigs served as a mixing vessel for the generation of reassortants with the precedent human H2N2 influenza virus [7-10]. To prepare for pandemic influenza, we have conducted a global surveillance of influenza in birds and mammals since 1977, and have established a vaccine strain library of influenza A viruses [11-15]. Their pathogenicity, antigenicity, genetic information, and yield in chicken embryos have been analyzed and the data are available at http://virusdb.czc.hokudai.ac.jp/.

In the present study, a vaccine strain against pandemic 2009 (H1N1) influenza was selected from 42 H1N1 influenza viruses in the virus library. The potency of inactivated whole virus particle (WV) and ether-split (ES) vaccines prepared from a virus strain in the library was evaluated.

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Results

Antigenic analysis of H1N1 influenza viruses

Eighteen H1N1 influenza virus strains were selected from 42 strains in the library, showing good growth in embryonated chicken eggs (data not shown). The 18 virus strains were antigenically analyzed by hemagglutination-inhibition (HI) test with chicken antisera to H1N1 viruses isolated from birds, pigs and humans (Table 1). The pandemic strain, A/Narita/1/2009 (H1N1) (Narita/09), which was the first isolate in Japan in 2009, reacted with the antiserum to Sw/Hok/81 at a titer of 1:640, 8-fold lower than that to homologous virus. The antiserum to Narita/09 reacted with swine influenza viruses, especially the isolates in 1930–1981 at a titer of 1:1,280-2,560, which was 2- to 4-fold lower than that to homologous virus. These results indicate that the antigenicity of Narita/09 was to some extent related to those of H1N1 classical swine flu virus strains.

Genetic analyses of H1N1 viruses

Nucleotide sequences of the HA genes of the 18 H1N1 viruses were phylogenetically analyzed by the neighbor-joining method with those of other H1N1 strains, including H1N1 viruses isolated from humans. Based on the results of phylogenetic analysis, H1 HA genes were grouped into human, swine, or avian origin clusters (Figure 1). Swine influenza viruses isolated in Japan during 1977–1981 were clustered with pandemic 2009 (H1N1) viruses. Identity of amino acid of HA between Sw/Hok/81 and Narita/09 was 89.9% and glycosylation sites of HA were not different.

Growth of H1N1 viruses in embryonated chicken eggs

The growth of 18 H1N1 viruses in embryonated chicken eggs was assessed. All the viruses replicated efficiently and had reached a plateau by 48 hours post-infection (p.i.). No significant difference in peak titers of vaccine candidates was detected (data not shown). Sw/Hok/81 showed the highest titer at $10^{8.3}$ plaque-forming units (PFU)/ml 48 hours p.i., which was 10 times higher than that of Narita/09 ($10^{7.3}$ PFU/ml).

Potency test of the vaccine against H1N1 pandemic virus in mice

Four, 20, and 100 μg protein of WV or ES vaccines of Narita/09 and Sw/Hok/81, respectively, were subcutaneously

Table 1 The cross-reactivity of H1N1 viruses isolated from pigs, humans, and birds

	HI titer of chicken antisera against representative H1 viruses					
Virusesa	Narita/09	Sw/Iowa/15/30	Sw/Hok/81	PR/8/34	Hok/4/96	Dk/Mong/540/01
A/Narita/1/2009	**5,120b**	80	640	40	40	80
Swine isolates						
A/swine/Iowa/15/1930	1,280	**1,280**	2,560	20	80	640
A/swine/Niigata/1/1977	1,280	1,280	2,560	40	160	640
A/swine/Shimane/1/1978	2,560	1,280	5,120	40	160	640
A/swine/Shizuoka/1/1978	2,560	1,280	5,120	40	160	640
A/swine/Toyama/1/1978	2,560	1,280	5,120	40	160	640
A/swine/Kanagawa/1/1978	1,280	1,280	640	40	320	640
A/swine/Hokkaido/2/1981	1,280	1,280	**5,120**	80	80	640
A/swine/Miyagi/5/2003 (H1N2)	640	320	2,560	160	80	80
Human isolates						
A/PR/8/1934	20	40	40	**2,560**	160	20
A/Hokkaido/2/1996	320	80	80	160	**5,120**	320
A/Hokkaido/11/2002	160	80	80	320	5,120	80
Avian isolates						
A/duck/Miyagi/66/1977	160	80	80	40	40	640
A/swan/Hokkaido/55/1996	320	80	40	80	80	1,280
A/duck/Hokkaido/1130/2001	160	80	40	<20	<20	1,280
A/duck/Hokkaido/1203/2001	160	80	80	<20	<20	640
A/duck/Mongolia/540/2001	80	160	40	<20	20	**1,280**
A/duck/Hokkaido/83/2004	160	80	40	<20	<20	640
A/duck/Hokkaido/W73/2007	80	80	80	<20	<20	640

a: Subtype of viruses was H1N1 exept for A/swine/Miyagi/5/2003 (H1N2).
b: Homologous titer was shown in bold.

Figure 1 Phylogenetic tree of H1 HA gene of influenza viruses. 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 boot strap analysis with 1,000 replications. Viruses shown in bold were used in this study and classified into 3 groups according to the animal from which they were isolated (human, avian and swine). Subtypes of viruses were H1N1 except for those in parentheses. Sw: swine, Dk: duck.

injected once into 5 mice. The serum antibody titers of mice against the vaccine and challenge strains were examined (Table 2). The neutralization (NT) antibodies were induced by each vaccine in a dose-dependent manner. Serum NT antibodies induced by injection of WV or ES vaccine of Sw/Hok/81 were not detected with Narita/09.

To assess the potency of the vaccine against the challenge with pandemic 2009 (H1N1) virus, $10^{6.0}$ PFU of Narita/09 were intranasally inoculated into mice which were injected subcutaneously once with each of the test vaccines. The rate of weight loss of the mice after virus challenge is shown in Figure 2. The mice injected with Narita/09 or Sw/Hok/81 vaccines survived for 14 days, although they showed some weight loss, while the non-vaccinated control mice showed significant weight loss and had died by day 14 after the challenge. In the mice injected with Narita/09 vaccine, no significant difference in weight loss was observed in the mice vaccinated with WV or ES vaccine. The mice injected with ES vaccine of Sw/Hok/81, however, showed significant weight loss compared with mice injected with WV vaccine. The rate of weight loss of mice injected with ES vaccine of Sw/Hok/81 correlated in a dose-dependent manner. The potency of vaccines was also evaluated by measuring the virus titer in the lower respiratory tract of mice (Table 2). The virus titers in the lungs were $10^{4.3}$–$10^{4.7}$ PFU/g in mice injected with

Table 2 Neutralizing antibody titers of mice injected once with the vaccine and virus titers in the lungs after challenge

Vaccine			NT titer to		Virus titer in lungs, mean log PFU/g ±Se[a]
Strain	Protein, μg	Formulation	Narita/09	Sw/Hok/81	
PBS	-	-	<10, <10, <10, <10, <10	<10, <10, <10, <10, <10	5.0±0.17
Narita/09	4	ES	<10, <10, <10, <10, <10	ND	4.6±0.10
	20	ES	<10, 20, 20, 20, 40	ND	4.6±0.12
	100	ES	20, 40, 80, 160, 160	ND	4.1±0.35
Narita/09	4	WV	40, 40, 40, 80, 160	ND	4.2±0.17
	20	WV	40, 80, 160, 160, 320	ND	3.9±0.25*
	100	WV	320, 320, 640, 1280, 1280	ND	2.4±0.50**
Sw/Hok/81	4	ES	<10, <10, <10, <10, <10	<10, <10, <10, 10, 10	4.6±0.04
	20	ES	<10, <10, <10, <10, <10	<10, 10, 10, 20, 80	4.4±0.02
	100	ES	<10, <10, <10, <10, <10	10, 20, 20, 40, 40	4.7±0.02
Sw/Hok/81	4	WV	<10, <10, <10, <10, <10	20, 40, 40, 40, 80	4.5±0.06
	20	WV	<10, <10, <10, <10, <10	20, 40, 80, 80, 80	4.4±0.04
	100	WV	<10, <10, <10, <10, <10	160, 160, 160, 160, 320	4.3±0.09

Mice were injected with each vaccine subcutaneously. Serum samples were collected 3 weeks after injection.
The animals were challenged by intranasal administration of 106.0 PFU of A/Narita/09.
At 3 days after challenge, lungs samples were collected and virus titers were measured. ES: ether split vaccine, WV: whole inactivated vaccine
a: Data are for 5 mice.
*: $P<0.05$, vs. virus titers in PBS group.
**: $P<0.01$, vs. virus titers in PBS group.

100, 20, and 4 μg protein of each vaccine of Sw/Hok/81, and $10^{5.0}$ PFU/g in the non-vaccinated mice.

To improve the efficacy of the Sw/Hok/81 vaccine, WV or ES vaccine of Sw/Hok/81 was injected twice into mice. At 2 weeks p.i., the serum NT antibody titers of the mice injected with the vaccine were higher than that of mice injected once (Table 3). Although the challenge appeared to be less severe compared to first experiment (Figure 3), the virus titers of the lungs of the mice were similar to those of mice injected once with Narita/09 vaccine (Table 3). These results indicate that even if an antigenic difference was observed between vaccine and challenge strains, the WV vaccine induced immunity in mice, decreasing the impact of disease caused by the challenge strain.

Discussion

Vaccination is a measure to reduce the impact of influenza; however, it takes 6 months to prepare a vaccine [16]. Virus isolates from humans usually do not grow well in embryonated chicken eggs, which poses significant limitations for influenza vaccine production. Attempts to increase the yield of candidate vaccine strains have been made by multiple passages in eggs over time or genetic reassortment with a high growth laboratory strain [17,18]. To prepare for pandemic influenza, a virus library of non-pathogenic influenza A viruses with 144 combinations of 16 HA and 9 NA subtypes has been established [15]. In the present study, we selected vaccine strains from 18 H1N1 virus isolates from birds, pigs, and humans on the basis of their growth in embryonated chicken eggs and their antigenicity. Among these viruses, the yield of Sw/Hok/81 in embryonated chicken eggs showed $10^{8.3}$ PFU/ml, which is higher than that of Narita/09 ($10^{7.3}$ PFU/ml), indicating that a virus strain selected from the influenza virus library could be used for the vaccine strain.

The 1957 and 1968 pandemic influenza virus strains were reassortants of avian and human strains [19]. Kida *et al.* showed that viruses in pigs are in antigenically stasis, as are those in ducks, compared with influenza viruses in humans [9,10]. The present results of antigenic analysis of H1N1 viruses indicate that pandemic 2009 (H1N1) virus was antigenically similar to that of classical swine influenza viruses, not to that of human influenza viruses, as previously described by Garten *et al.* [2]. Although we cannot predict the subtype of the pandemic strain, the antigenicity of the virus is conserved in pigs or ducks. Thus, antigenically related strains isolated from natural hosts could be used for human pandemic influenza vaccines. In order to update the influenza virus library as a seed of vaccine strains, continuous surveillance of avian and swine influenza and the study of pathogenicity, antigenicity, genetic information, and yield in chicken embryo of virus strains are needed.

In the present study, to prepare for future pandemics, we evaluated the potency of a vaccine prepared from Sw/Hok/81 against the pandemic 2009 (H1N1) virus. It was revealed that mice injected with WV or ES vaccine prepared from Sw/Hok/81 induced immunity to suppress the disease manifestation after challenge with Narita/09, although an

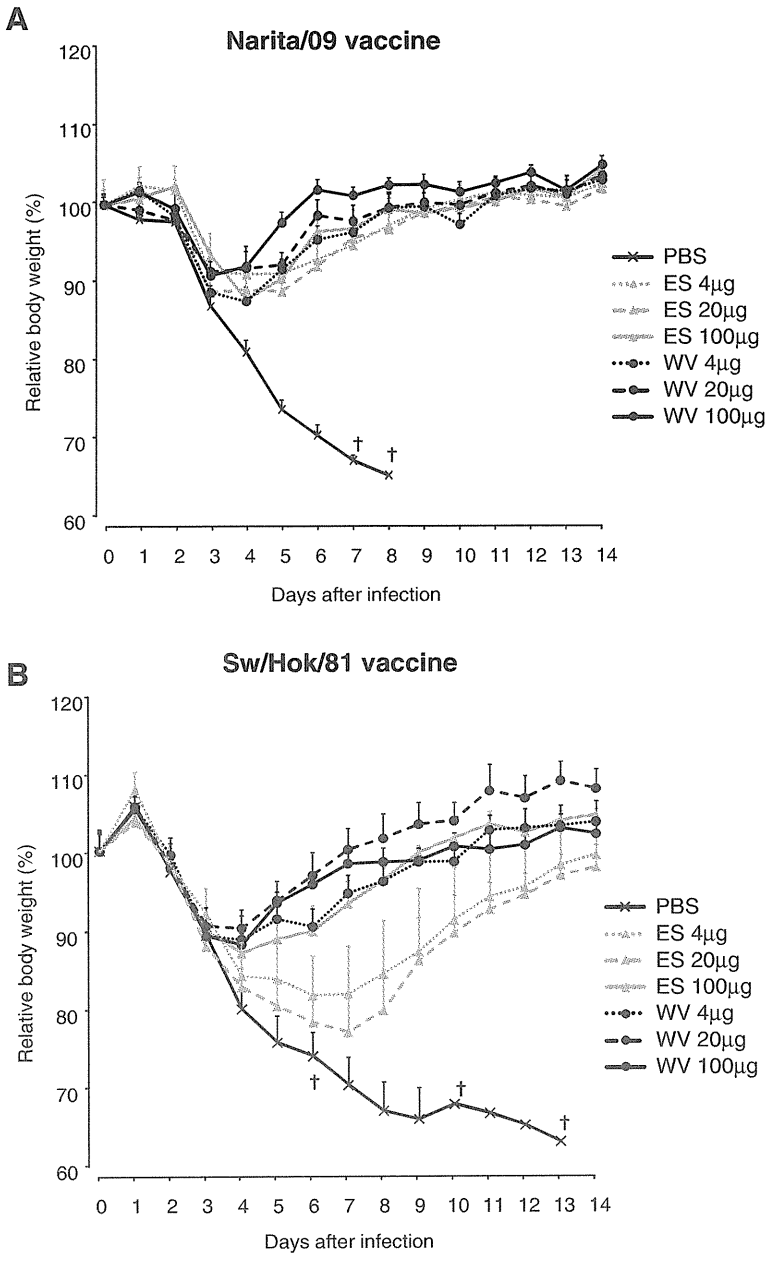

Figure 2 Changes in body weight of mice injected subcutaneously once with Narita/09 (A) or Sw/Hok/81 (B) vaccine after the challenge with Narita/09. Data are shown as mean body weight ± standard error. ES: ether split, WV: whole inactivated. †: Mice died.

antigenic difference was observed in these viruses. WV vaccine induces higher immune responses after intramuscular immunization and is superior to ES and subunit vaccine in human populations [20,21]. The reason for these immune responses to WV vaccine is the stimulation of innate [22] and cell-mediated immune responses to internal viral proteins. Indeed, identity of NP protein between Sw/Hok/81 and Narita/09 were 96.9%. In the previous studies, WV vaccine prepared from a virus strain selected from the library also showed protective efficacy against H5 and H7 virus infection in chicken, mice and cynomolgus macaques [23-28]. These results suggest that WV vaccine should work best in immunologically naive people in the early phase of a pandemic and two injections of the vaccine will be more effective even if the antigenicity of the pandemic strain is partially different from the vaccine strain.

Conclusion

The potency of the vaccine prepared from Sw/Hok/81 for the pandemic 2009 (H1N1) virus was evaluated. Mice injected once with WV vaccine prepared from Sw/Hok/81 induced immunity to suppress weight loss and virus growth

Table 3 Neutralizing antibody titers of mice injected twice with the vaccine and virus titers in the lungs after challenge

Vaccine			NT titer to		Virus titer in lungs, mean log PFU/g ±Se[a]
Strain	Protein, μg	Formulation	Narita/09	Sw/Hok/81	
PBS	-	-	<10, <10, <10, <10, <10	<10, <10, <10, <10, <10	4.4±0.08
Sw/Hok/81	4.0	ES	<10, <10, <10, <10, <10	20, 40, 80, 160, 160	4.4±0.07
	20	ES	<10, <10, <10, <10, <10	80, 80, 160, 160, 160	4.2±0.19
	100	ES	<10, <10, <10, <10, <10	80, 80, 160, 320, 310	3.9±0.14*
Sw/Hok/81	4.0	WV	<10, <10, <10, <10, <10	160, 320, 320, 640, 640	4.2±0.11
	20	WV	<10, <10, <10, <10, <10	160, 320, 640, 640, 640	3.9±0.28
	100	WV	<10, 10, 40, 40, 160	160, 320, 640, 640, 640	2.9±0.30**

Mice were injected twice with each vaccine subcutaneously with a 2-week interval. Serum samples were collected 2 weeks after the final immunization.
The animals were challenged by intranasal administration of 106.0 PFU of A/Narita/09.
At 3 days after challenge, lungs samples were collected and virus titers were measured. ES: ether split vaccine, WV: whole inactivated vaccine
a: Data are for 5 mice.
*: P<0.05, vs. virus titers in PBS group.
**: P<0.01, vs. virus titers in PBS group.

in the lungs after challenge with Narita/09. The suppression of virus recovery from lungs of mice injected twice with WV vaccine was similar to that in mice injected once with Narita/09 vaccine. These results suggest that WV vaccine should work best in immunologically naive people in the early phase of a pandemic, and two injections of the vaccine will be more effective if the antigenicity of the pandemic strain is partially different from the vaccine strain.

Materials and methods

Viruses and cells

Eighteen H1N1 influenza viruses isolated from humans, pigs and wild birds were used in the present study as representative of 42 H1N1 virus strains in our virus library (http://virusdb.czc.hokudai.ac.jp/). Narita/09 was provided by the National Institute of Infectious Diseases (Tokyo, Japan). Viruses were propagated in 10-day-old embryonated chicken eggs at 35°C for 48 hours.

Madin-Darby canine kidney (MDCK) cells were maintained in minimum essential medium (Nissui, Japan) supplemented with calf serum and used for titration of viral infectivity.

Sequencing and phylogenetic analysis

Viral RNA was extracted with TRIzol LS reagent (Invitrogen, Carlsbad, CA, USA) from the allantoic fluid of chicken

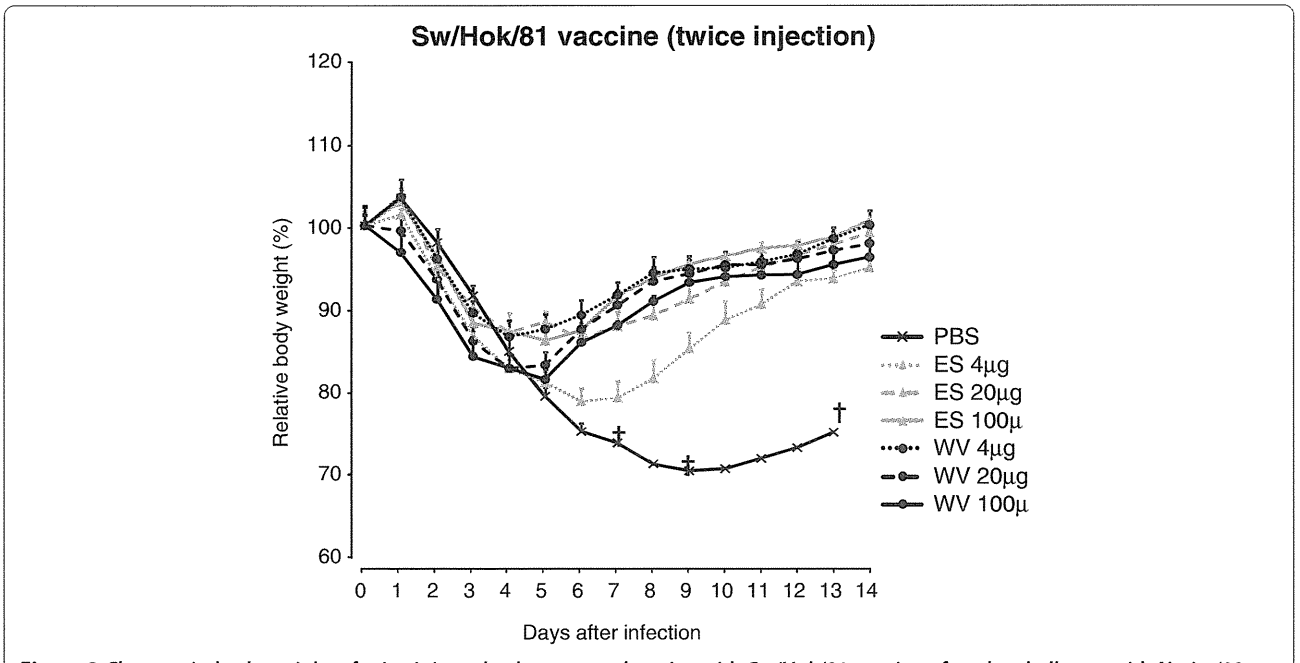

Figure 3 Changes in body weight of mice injected subcutaneously twice with Sw/Hok/81 vaccine after the challenge with Narita/09. Data are shown as mean body weight ± standard error. ES: ether split, WV: whole inactivated. †: Mice died.

embryos infected with the virus. Nucleotide sequences of all eight gene segments were determined after RT-PCR, as described previously [29]. The sequence data were analyzed using GENETYX ver. 9.1 (GENETYX Corporation, Tokyo, Japan). Phylogenetic analysis of the HA gene was performed by BioEdit ver. 7.0 and MEGA 5 by the neighbor-joining method with 1,000 bootstraps.

Serological tests

HI tests were performed by the microtiter method [30]. The HI titer was expressed as the reciprocal of the highest serum dilution showing complete inhibition of the hemagglutination of 4 HA units of the virus. In NT tests, titers were determined as the reciprocals of serum dilution of the complete inhibition of the cytopathic effect of 100 PFU of viruses using MDCK cells.

Viral growth in embryonated chicken eggs

Viruses of 100 50% egg infectious dose (EID_{50}) were inoculated into 10-day-old embryonated chicken eggs and incubated at 35°C for 48 hours. Allantoic fluid was harvested to determine viral titers at different time points (0, 12, 24, 48, and 72 hr). The PFU of each virus in the allantoic fluid was determined.

Vaccine preparation

To assess the potency of vaccines, inactivated WV vaccines of Sw/Hok/81 and Narita/09 were prepared as described previously [31]. ES vaccine of each strain was also prepared according to Kida *et al.* [32]. Briefly, purified viruses were disrupted with 0.1% Tween 80 and an equal volume of diethyl-ether for 30 min at room temperature. After centrifugation for 30 min at 6,000 g, the water phase was collected and ether dissolved in water was blown out with a stream of nitrogen.

Potency test of vaccine against Narita/09 in mice

WV or ES vaccines of each strain with 4, 20 and 100 μg protein were injected subcutaneously into ten 4-week-old female BALB/c mice (CLEA Japan Inc., Tokyo, Japan), respectively. PBS was injected into control mice. Three weeks after immunization, serum samples were collected and 30 μl of $10^{6.0}$ PFU of Narita/09 was intranasally inoculated into the mice under anesthesia. Three days after the challenge, five mice in each group were sacrificed and the lungs were collected. The virus titers in the lung homogenates were quantified by plaque assay of MDCK cells. Five other mice were observed for clinical signs and weight loss for 14 days. WV and ES vaccines of Sw/Hok/81 were also injected into mice twice with a 2-week interval. Two weeks after the final injection, the serum samples were collected and Narita/09 was inoculated into mice. Animal experiments were authorized by the Institutional Animal Care and Use Committee of the Graduate School of Veterinary Medicine, Hokkaido University (approved numbers: 9148 and 1052) and all experiments were performed according to the guidelines of this committee.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MO drafted the manuscript and prepared the vaccines used in the present study. MO, TH, NM carried out animal experiment. YS, and HK participated in the coordination of the study. All authors read and approved the final manuscript.

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VIROLOGY JOURNAL

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The PB2, PA, HA, NP, and NS genes of a highly pathogenic avian influenza virus A/whooper swan/Mongolia/3/2005 (H5N1) are responsible for pathogenicity in ducks

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Abstract

Background: Wild ducks are the natural hosts of influenza A viruses. Duck influenza, therefore, has been believed inapparent infection with influenza A viruses, including highly pathogenic avian influenza viruses (HPAIVs) in chickens. In fact, ducks experimentally infected with an HPAIV strain, A/Hong Kong/483/1997 (H5N1) (HK483), did not show any clinical signs. Another HPAIV strain, A/whooper swan/Mongolia/3/2005 (H5N1) (MON3) isolated from a dead swan, however, caused neurological dysfunction and death in ducks.

Method: To understand the mechanism whereby MON3 shows high pathogenicity in ducks, HK483, MON3, and twenty-four reassortants generated between these two H5N1 viruses were compared for their pathogenicity in domestic ducks.

Results: None of the ducks infected with MON3-based single-gene reassortants bearing the PB2, NP, or NS gene segment of HK483 died, and HK483-based single-gene reassortants bearing PB2, NP, or NS genes of MON3 were not pathogenic in ducks, suggesting that multiple gene segments contribute to the pathogenicity of MON3 in ducks. All the ducks infected with the reassortant bearing PB2, PA, HA, NP, and NS gene segments of MON3 died within five days post-inoculation, as did those infected with MON3. Each of the viruses was assessed for replication in ducks three days post-inoculation. MON3 and multi-gene reassortants pathogenic in ducks were recovered from all of the tissues examined and replicated with high titers in the brains and lungs.

Conclusion: The present results indicate that multigenic factors are responsible for efficient replication of MON3 in ducks. In particular, virus growth in the brain might correlate with neurological dysfunction and the disease severity.

Keywords: H5N1 influenza virus, Duck, Natural host, Pathogenicity

Background

Influenza A viruses have eight-segmented, negative, and single-stranded RNA genomes and are serologically divided into 16 hemagglutinin (HA) (H1-H16) and 9 neuraminidase (NA) (N1-N9) subtypes [1,2]. Influenza A viruses are widely distributed in birds and mammals, including humans. Ecological studies have revealed that wild waterbirds, especially migratory ducks, are the natural hosts of influenza A viruses. Each of the known subtypes of influenza A virus has been perpetuated among water birds and in the water of the lakes where they nest in summer [3,4]. Furthermore, influenza A viruses circulating in nature are nonpathogenic in ducks and evolutionarily static [5,6], suggesting that the viruses and hosts have reached a long-established adaptive optimum.

Influenza A viruses maintained in ducks usually do not transmit to and infect chickens directly. It is known that low pathogenic viruses occasionally infect chickens after passage in domestic water birds such as ducks and

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geese and terrestrial birds such as quails and turkeys, and then may acquire high pathogenicity in chickens through multiple transmissions in the chicken population [7]. Highly pathogenic avian influenza viruses (HPAIVs) are so far restricted to H5 and H7 viruses, although most of the viruses of these subtypes are not highly pathogenic in chickens [8]. In 1997, outbreaks of highly pathogenic avian influenza (HPAI) occurred at live bird markets in Hong Kong and human cases of infection with the H5N1 virus were found [9,10]. Since then, H5N1 HPAIVs have thus been circulating in poultry for more than a decade [11].

It was generally thought that ducks could tolerate infection with influenza A viruses, including HPAIVs. In fact, A/Hong Kong/483/1997 (H5N1) (HK483) was not lethal for experimentally infected ducks [12]; however, in 2002, a large number of water birds, including ducks, geese, and other birds, died due to infection with H5N1 HPAIVs in Hong Kong [13]. In 2005, approximately six thousand migratory water birds were found dead with H5N1 virus infection in Qinghai Lake, China [14,15] and then virus strains of this subtype have been isolated in the Middle East, Europe, and Africa [16]. Since 2005, H5N1 HPAIVs originating from southern China have been isolated from dead aquatic birds such as swans and geese on their migratory routes to the northern nesting lakes in spring in Japan, Mongolia, and Russia [17-19]. It was reported that ducks experimentally infected with A/whooper swan/Mongolia/3/2005 (H5N1) (MON3) showed neurological dysfunction and died [17]. The mechanism whereby these H5N1 HPAIVs show high pathogenicity in ducks is still unclear.

In chickens, it is well known that insertion of a pair of dibasic amino acid residues at the cleavage site of the HA renders avian influenza viruses capable of infecting endothelial cells, followed by systemic infection [20-22]. Both HK483 and MON3 have multiple basic amino acid residues at the HA cleavage site, representing their high pathogenicity in chickens. The difference in pathogenicity in ducks between HK483 and MON3 hence suggests that factors other than the cleavage activation of the HA molecule contribute to the pathogenicity in ducks. To understand the molecular basis of the high pathogenicity of MON3 in ducks, reassortants were generated between HK483 and MON3 by reverse genetics and compared for their pathogenicity in domestic ducks. The present results indicate that multigenic factors were involved in the high pathogenicity of MON3 in ducks.

Results

Pathogenicity of H5N1 viruses in ducks of different ages

To investigate whether the age of ducks affects their susceptibility to H5N1 virus infection, either HK483 or MON3 was inoculated intranasally into 1-day-, 2-week-, and 4-week-old domestic ducks and clinical symptoms were monitored up to 14 days post-inoculation (dpi). None of the ducks inoculated with HK483 showed clinical signs for 14 days. On the other hand, all of the 1-day- and 2-week-old ducks inoculated with MON3 died by 3 and 7 dpi, respectively (Table 1). They showed depression and convulsion and some of the 2-week-old ducks showed torticollis. In 4-week-old ducks, two of the three birds infected with MON3 survived, although they showed depression, torticollis, and blindness. On 3 dpi, 1-day-, 2-week-, and 4-week-old ducks infected with either HK483 or MON3 were euthanized and lung and brain samples were collected to assess virus replication in ducks at different ages (Table 1). HK483 was recovered from each of the tissues tested from 1-day-old ducks; however, replication of HK483 was restricted in 2- and 4-week-old ducks; HK483 was not detected either in the brains of 2-week-old ducks or in the brains, tracheas, and livers of 4-week-old ducks (data not shown). On the other hand, MON3 was recovered from each of the tissues of ducks experimentally infected regardless of their age. As shown with high titers in the brains and lungs, both viruses replicated more efficiently in 1-day- and 2-week-old ducks than 4-week-old ducks (Table 1). The present data demonstrate that younger ducks were more susceptible to HK483 and MON3 than older ducks. In the following experiments, 2-week-old ducks were adopted, since obvious difference was found in mortality and virus replication in their brains between HK483 and MON3 infection.

Gene segments responsible for pathogenicity of MON3 in ducks

In order to identify the gene segments responsible for the high pathogenicity of MON3 in ducks, MON3-based reassortants bearing a single segment of HK483 and the other seven segments of MON3 (HK483 PB2/MON3,

Table 1 Comparison of pathogenicity of HK483 and MON3 in 1-day-, 2-week-, and 4-week-old ducks

Virus	Age of ducks	Mortality[a]	MDD[b] (range)	Virus titers[c]	
				Brain	Lung
HK483	1 day	0/3	≥ 14	5.4, 2.5, 2.5	5.5, 4.5, 2.5
	2 weeks	0/6	≥ 14	-, -, -[d]	4.3, 3.5, 2.5
	4 weeks	0/3	≥ 14	-, -, -	2.5, -, -
MON3	1 day	9/9	2.2 (2–3)	8.0, 7.5	5.7, 5.5
	2 weeks	10/10	4.9 (4–7)	8.8, 7.3, 7.3	8.5, 5.5, 5.3
	4 weeks	1/3	7.0	5.5, 4.5, -	4.5, 2.5, 3.7

[a] Number of dead ducks/number of examined ducks.
[b] Mean death days post-inoculation.
[c] At 3 days post-inoculation, the brains and lungs were collected. Suspension of each tissue sample was inoculated into 10-day-old embryonated eggs for titration. Virus titers in tissues were expressed as log 50% egg infectious dose (EID_{50})/g. The lower limit of detection was $10^{1.5}$ EID_{50}/g.
[d] -: < 1.5 log EID_{50}/g.

HK483 PB1/MON3, HK483 PA/MON3, HK483 HA/MON3, HK483 NP/MON3, HK483 NA/MON3, HK483 M/MON3, and HK483 NS/MON3) were inoculated intranasally into three 2-week-old ducks to assess their pathogenicity. All ducks infected with HK483 NA/MON3 or HK483 M/MON3 showed severe depression and died on 5 to 7 dpi, as did ducks infected with MON3 (Figure 1). Pathogenicity of HK483 PB1/MON3, HK483 PA/MON3, and HK483 HA/MON3 in ducks was slightly lower than MON3 (i.e., one of three ducks infected with each reassortant survived for 14 days), although all ducks showed depression and the surviving duck infected with HK483 PB1/MON3 developed severe torticollis. HK483 PB2/MON3, HK483 NP/MON3, and HK483 NS/MON3 did not exhibit lethality in ducks, whereas some of the ducks infected with these reassortants showed lethargy and/or mild torticollis (Figure 1). Similarly, HK483-based reassortants bearing a single segment of MON3 and the other seven segments of HK483 (MON3 PB2/HK483, MON3 PB1/HK483, MON3 PA/HK483, MON3 HA/HK483, MON3 NP/HK483, MON3 NA/HK483, MON3 M/HK483, and MON3 NS/HK483) were inoculated into three 2-week-old ducks. Although some ducks showed mild lethargy, all the ducks survived for the 14-day observation period (Figure 1). These results revealed that the PB2, NP, and NS gene segments of MON3 were prerequisite for high pathogenicity in ducks; however, MON3 PB2/HK483, MON3 NP/HK483, and MON3 NS/HK483 did not cause severe clinical symptoms in any ducks, suggesting that multiple gene factors are involved in the high pathogenicity of MON3 in ducks.

To clarify the minimum set of MON3 gene segments required for its high pathogenicity in ducks, eight multi-gene reassortants between HK483 and MON3 were compared for their pathogenicity in ducks (Figure 2). These multi-gene reassortants uniformly possessed the MON3 PB2, NP, and NS gene segments prerequisite for high pathogenicity in ducks and the HK483 M and NA gene segments which unlikely contribute to the pathogenicity in ducks. These multi-gene reassortants had 8 possible combinations of the PB1, PA, and HA gene segments from HK483 and MON3 (Figure 2). These reassortants were designated MON3 PB2-NP-NS/HK483, MON3 PB2-PB1-NP-NS/HK483, MON3 PB2-PA-NP-NS/HK483, MON3 PB2-HA-NP-NS/HK483, MON3 PB2-PB1-PA-NP-NS/HK483, MON3 PB2-PB1-HA-NP-NS/HK483, MON3 PB2-PA-HA-NP-NS/HK483, and MON3 PB2-PB1-PA-HA-NP-NS/HK483. None of the ducks infected with MON3 PB2-NP-NS/HK483, MON3 PB2-PB1-NP-NS/HK483, or MON3 PB2-PA-NP-NS/HK483 manifested any clinical symptoms (Figure 2), except for a duck which showed lethargy upon MON3 PB2-NP-NS/HK483 infection. All the ducks inoculated with MON3 PB2-HA-NP-NS/HK483 showed depression and one of the three ducks died on 6 dpi. Following depression and cyanosis, all the ducks infected with MON3 PB2-PA-HA-

Virus	Origin of gene segment								Mortality*	MDD**
	PB2	PB1	PA	HA	NP	NA	M	NS		
HK483	□	□	□	□	□	□	□	□	0/6	14≤
MON3	■	■	■	■	■	■	■	■	7/7	4.9 (4-7)
HK483 PB2/MON3	□	■	■	■	■	■	■	■	0/3	14≤
HK483 PB1/MON3	■	□	■	■	■	■	■	■	2/3	6.0 (5,7)
HK483 PA/MON3	■	■	□	■	■	■	■	■	2/3	8.0 (6,10)
HK483 HA/MON3	■	■	■	□	■	■	■	■	2/3	6.5 (6,7)
HK483 NP/MON3	■	■	■	■	□	■	■	■	0/3	14≤
HK483 NA/MON3	■	■	■	■	■	□	■	■	3/3	5.7 (5-7)
HK483 M/MON3	■	■	■	■	■	■	□	■	3/3	5.7 (5-6)
HK483 NS/MON3	■	■	■	■	■	■	■	□	0/3	14≤
MON3 PB2/HK483	■	□	□	□	□	□	□	□	0/3	14≤
MON3 PB1/HK483	□	■	□	□	□	□	□	□	0/3	14≤
MON3 PA/HK483	□	□	■	□	□	□	□	□	0/3	14≤
MON3 HA/HK483	□	□	□	■	□	□	□	□	0/3	14≤
MON3 NP/HK483	□	□	□	□	■	□	□	□	0/3	14≤
MON3 NA/HK483	□	□	□	□	□	■	□	□	0/3	14≤
MON3 M/HK483	□	□	□	□	□	□	■	□	0/3	14≤
MON3 NS/HK483	□	□	□	□	□	□	□	■	0/3	14≤

Figure 1 Pathogenicity of HK483, MON3, and single-gene reassortants in 2-week-old ducks. HK483, MON3, and a series of reassortants between HK483 and MON3 were generated by reverse genetics. Each of the viruses was inoculated into 2-week-old ducks and clinical symptoms of challenged ducks were observed over 14 dpi. White and gray boxes indicate the derivation of virus gene segments from HK483 and MON3, respectively. *: number of dead birds/number of examined ducks. **: mean death dpi.

Virus	Origin of gene segment								Mortality*	MDD** (range)
	PB2	PB1	PA	HA	NP	NA	M	NS		
MON3 PB2-NP-NS/HK 483	■	□	□	□	■	□	□	■	0/5	14≤
MON3 PB2-PB1-NP -NS/HK483	■	■	□	□	■	□	□	■	0/3	14≤
MON3 PB2-PA-NP -NS/HK483	■	□	■	□	■	□	□	■	0/3	14≤
MON3 PB2-HA-NP -NS/HK483	■	□	□	■	■	□	□	■	1/3	6.0
MON3 PB2-PB1-PA -NP-NS/HK483	■	■	■	□	■	□	□	■	1/3	8.0
MON3 PB2-PB1-HA -NP-NS/HK483	■	■	□	■	■	□	□	■	1/3	6.0
MON3 PB2-PA-HA -NP-NS/HK483	■	□	■	■	■	□	□	■	3/3	5.0 (5)
MON3 PB2-PB1-PA -HA-NP-NS/HK483	■	■	■	■	■	□	□	■	3/3	6.7 (6-7)

Figure 2 Pathogenicity of multi-gene reassortants in 2-week-old ducks. A series of multi-gene reassortants between HK483 and MON3 was generated by reverse genetics. Each of the viruses was inoculated into 2-week-old ducks and clinical symptoms of challenged ducks were observed over 14 dpi. White and gray boxes indicate the derivation of virus gene segments from HK483 and MON3, respectively. *: number of dead birds/number of examined ducks. **: mean death dpi.

NP-NS/HK483 died within 5 dpi as did ducks infected with MON3 (Figures 1 and 2). These results indicate that PB2, PA, HA, NP, and NS gene segments of MON3 were the required minimum set to show full pathogenicity in ducks.

Replication of reassortants in 2-week-old ducks

To further compare viral replication and distribution in ducks of HK483, MON3, and reassortants, virus titers in various tissue samples of ducks infected with these viruses were estimated on 3 dpi. Mean titers of MON3 were more than one hundred times as high as those of HK483 in each of the tissues tested (Figure 3). In particular, MON3 was detected at the highest titers in the brains among the tissues examined, while HK483 was not recovered from the brains. Dissemination of single- and multi-gene reassortants in 2-week-old ducks was also assessed on 3 dpi (Figures 4 and 5). Viruses that were lethal to ducks were recovered from various tissues and replicated with high titers especially in the brains. On the other hand, viruses that caused no disease in ducks replicated in restricted organs such as the lungs, kidneys, and colon. These data suggested that acute and efficient viral replication in systemic organs including the brain was the cause of death accompanying neurological symptoms.

Discussion

Since the outbreaks of HPAI in waterbirds occurred in Hong Kong in 2002, a large number of free-flying water birds have died due to H5N1 HPAIV infection in the last decade; however, little is known about the mechanism whereby these viruses show high pathogenicity in ducks. It has been reported that tyrosine at position 436 of PB1 and threonine at position 516 of PA were associated with high pathogenicity in ducks [23]. Three amino acid residues in PB1-F2 were also reported as determinants of pathogenicity of an H5N1 HPAIV in mallards [24]. Recently, Song *et al.* [25] demonstrated that proline and aspartic acid at positions 224 and 383, respectively, of

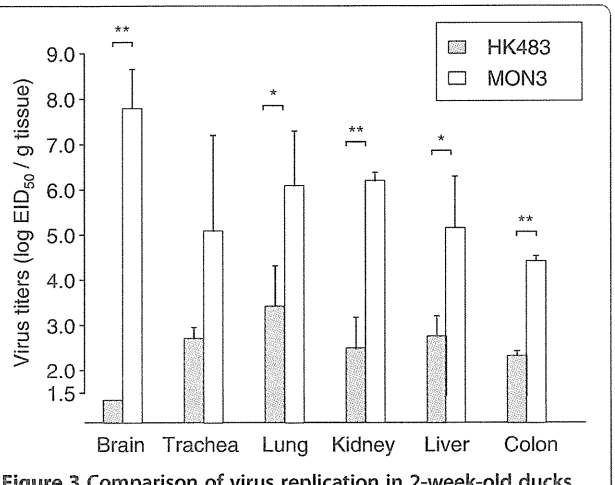

Figure 3 Comparison of virus replication in 2-week-old ducks between HK483 and MON3. Three days post-inoculation, the brains, tracheas, lungs, kidneys, livers, and colons were collected. Viruses in each of the tissue samples were titrated in 10-day-old embryonated chicken eggs and titers were expressed as EID_{50}/g of tissues. The mean virus titers in each of the tissues and standard deviations are shown. The lower limit of detection was $10^{1.5}$ EID_{50}/g. Virus titers were statistically analyzed by Student's paired *t*-test. Significant differences are indicated by asterisks (** $p < 0.001$, * $p < 0.05$).